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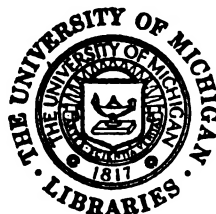
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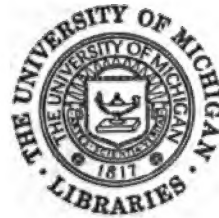
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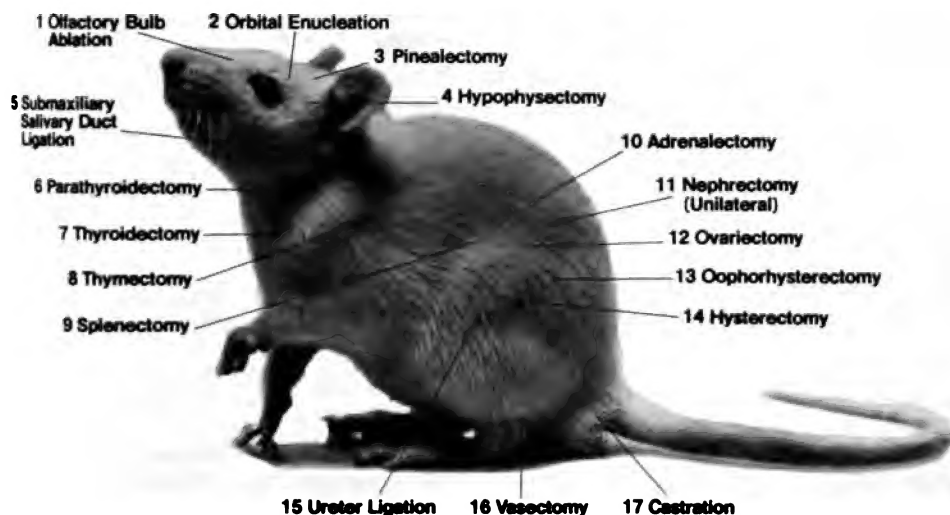
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Volume 159, Number 1, October 1978

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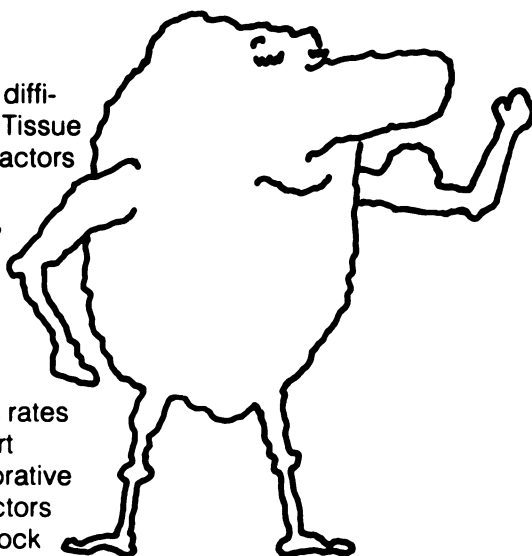
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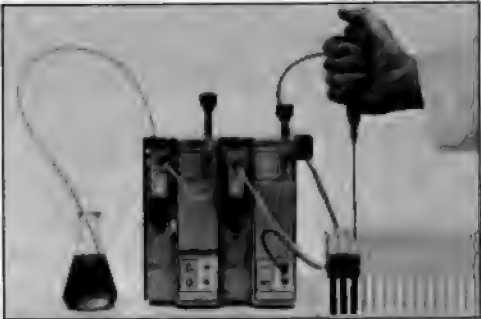


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# Effects of Testosterone Induced-Cholesterol Synthesis in Rat Ventral Prostate (40271)

ANIL K. SINGHAL, DANIEL P. BONNER AND CARL P. SCHAFFNER

From the Institute of Microbiology, Rutgers-The State University of New Jersey, New Brunswick, New Jersey 08903

It has been well established that under conditions testosterone administration significantly affects the rates of DNA, lipid and protein synthesis in the rat ventral prostate (1-4). Testosterone also maintains morphology and secretory activity of the prostate gland, both in *in vitro* and *in vivo* studies (5-7). On castration, there is rapid regression of the rat ventral prostate including cessation of secretory function (8). Cholesterol has been found to be one of the constituents of the prostate secretion.

In this paper, we are reporting the regulation of cholesterol synthesis by testosterone in the rat ventral prostate. Kinetics of cholesterol synthesis in the ventral prostate during testosterone administration to castrated rats was studied in relation to prostate weight gain, DNA and protein synthesis.

**Materials and methods. Animals.** Groups of 10 male intact and castrated Wistar rats (250 g) were maintained on Purina rat chow and water *ad libitum* and were kept on alternating 12-hr light and 12-hr dark cycle. At necropsy final body weights were determined.

**Administration of testosterone to castrated rats.** Castrated animals were injected subcutaneously with 2 mg of testosterone propionate dissolved in sesame oil (10 mg/ml), at the same time every day for different periods up to 14 days.

***In vitro* incorporation of radioactive precursors into cholesterol, proteins and DNA by prostate tissues.** At various time intervals up to 14 days animals were anesthetized by intraperitoneal injections of sodium barbital and sacrificed by exsanguination. The lobes of the ventral prostate gland were freed of the fat covering. The tissues were minced and weighed immediately in pre-weighed test tubes and kept in ice until use. Approximately 25-35 mg of minced tissues were used to study the regulation of radioactive precursors into cholesterol, proteins and DNA.

The radioactive precursors, 2-[<sup>14</sup>C]acetate (sp. activity 50.3 mCi/mmol), 4,5-<sup>3</sup>H-L-leucine (sp. activity 5 Ci/mmol), and <sup>3</sup>H-methylthymidine (sp. activity 6.7 Ci/mmol) were used in these studies to determine their incorporation into cholesterol, protein and DNA, respectively. Tissues were incubated with 2 ml of Hank's Balanced Salt solution supplemented with 0.2% glucose and either 1 μCi/ml of 2-[<sup>14</sup>C]acetate or 1 μCi/ml of <sup>3</sup>H-leucine or 3 μCi/ml of <sup>3</sup>H-thymidine (pre-gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) at 37° for 2 hr on a constant speed shaker. At the end of the incubation period, the reaction was terminated by instant freezing of the tubes in a dry ice-acetone bath. The radioactivity of cholesterol, protein and DNA in the tissues was then determined.

**Analysis of radioactivity in cholesterol.** The tissues were saponified by the addition of alcoholic KOH to a final concentration of 10% KOH and 50% ethanol (95%) at 75° for 75 min. Unsaponified lipids were pooled by repeated extractions with *n*-hexane. The hexane extracts were evaporated under nitrogen and digitonin precipitation was carried out according to the procedure of Sperry (11). The cholesterol-digitonin complex was dissolved in 1 ml of methanol and 0.1 ml aliquots were counted in duplicate for [<sup>14</sup>C]activity in a Packard Scintillation Counter. The rates of synthesis were expressed as counts per minute per μg of prostatic DNA.

**Analysis of radioactivity in protein and DNA.** The tissues were homogenized with a Brinkmann polytron and crude protein or DNA was precipitated with 5 ml of 6% trichloroacetic acid (TCA) at 0°. After 10 min, the samples were centrifuged at 4°, and the precipitates were washed twice with 5 ml of 6% cold TCA. The precipitates were then extracted repeatedly with 95% ethanol:chloroform (3:1 v/v) to remove lipids. For radioactivity counting in proteins, the ethanol-chloroform extracted precipitates were dissolved in 2 ml of 10% NaOH and 0.2

ml aliquots were counted in duplicate for  $^3\text{H}$ -activity. To measure the incorporation of  $^3\text{H}$  thymidine into DNA, ethanol-chloroform extracted precipitates were dissolved in 2 ml of 0.3 N KOH at  $37^\circ$  for 60 min. Proteins and DNA were then reprecipitated from supernatants with 8 ml of 6% TCA. The KOH extraction and the TCA precipitation were repeated. The final TCA insoluble fraction was treated with 2 ml of 16% perchloric acid (PCA) for 20 min at  $70^\circ$ , followed by centrifugation. Aliquots (0.2 ml) of the acid-soluble fraction were counted in duplicate for determination of radioactivity in the DNA. All tritium determinations were made in a xylene based scintillation cocktail (aquasol-2, New England Nuclear) and counted in a Packard Scintillation Counter.

**Colorimetric determinations.** The amount of cholesterol was quantitated by first saponifying the tissues and the unsaponified fractions were used for digitonin precipitation. The cholesterol-digitonin complex was used for colorimetric determination by the method of Parekh and Jung (12).

DNA and proteins were extracted in similar manner described in the section above and colorimetric analyses were carried out employing the method of Abraham *et al.* (13) for DNA and the Biuret procedure (14) for protein assay.

**Results.** As expected, the data in Table I confirm that on castration the prostate weight declines to about 12% of the normal rat prostate weight. Body weights are not significantly affected. Amounts of cholesterol, protein and DNA in rat prostate gland, quantitated colorimetrically, also decline to 12%, 13% and 25% of their respective normal values. Rates of synthesis of cholesterol and DNA per  $\mu\text{g}$  prostatic DNA also decline to

about 8% and 5%, respectively, in castrated animals. Contrary to the decreases in rates of synthesis of DNA and cholesterol, the rate of protein synthesis per  $\mu\text{g}$  prostatic DNA remains constant in the castrated rats even though the total amount of DNA present in the prostate gland of castrated rats is significantly lower than in normal animals. This may be due to the synthesis of hydrolytic enzymes that would hydrolyze the prostatic DNA present in the normal gland. The results indicate that testosterone produced by the hypothalamus is essential for the maintenance of glandular cholesterol synthesis in the prostate among other prostatic functions.

To examine whether testosterone could restore the prostate cholesterol levels as the levels of macromolecules, 2 mg testosterone propionate in sesame oil (2 mg/ml) was injected daily subcutaneously in castrated rats for varying periods of 2 to 12 days. Prostate weight as well as cholesterol, DNA and protein content in the prostate gland were quantitated and the results are presented in Fig. 1. Administration of testosterone to castrated rats increased the amount of cholesterol in the prostate gland. DNA and protein content also increased. Prostate weights and the amounts of cholesterol and protein increased more significantly after 2 days of testosterone administration. The amount of DNA remained constant up to 2 days and increased significantly after 5 days of testosterone injections. All the parameters tested increased almost equally between 2 and 5 days of testosterone treatment. The amount of protein increases sharply after 5 days of treatment which is followed by steep increases in prostate weight and the amount of cholesterol. Contrary to the increases in prostate weight and in the

TABLE I. EFFECT OF CASTRATION ON THE SYNTHESIS OF PROSTATE CHOLESTEROL, PROTEIN AND DNA

Type	Body weight (gm)	Wet prostate weight (mg) <sup>a</sup>	Prostate Cholesterol		Prostate Protein		Prostate DNA
			Total content ( $\mu\text{g}$ ) <sup>a</sup>	Rate of synthesis (cpm) <sup>b</sup>	Total content (mg) <sup>a</sup>	Rate of synthesis (cpm) <sup>b</sup>	Total content ( $\mu\text{g}$ ) <sup>a</sup>
Normal	275.5	116.02	237.5	46.62	5.83	108.91	293.17
	$\pm 11.5$	$\pm 21.06$	$\pm 48.1$	$\pm 11.98$	$\pm 0.983$	$\pm 70.49$	$\pm 57.97$
Castrated <sup>c</sup>	256.0	17.75	32.04	3.816	0.728	102.62	78.38
	$\pm 16.1$	$\pm 1.26$	$\pm 9.46$	$\pm 0.642$	$\pm 0.106$	$\pm 33.07$	$\pm 12.43$

<sup>a</sup> All the total contents are expressed in terms of per 100 g body wt.

<sup>b</sup> The rate of synthesis are expressed as cpm/ $\mu\text{g}$  of prostate DNA isolated.

<sup>c</sup> Rats were castrated for 7 days.

f cholesterol, the slopes of curves for DNA and protein at 12 days of testosterone administration approached the steady state.

In Fig. 2 the ratios of cholesterol, protein and DNA content of the prostate glands from

testosterone treated castrated rats are presented. The ratios of both cholesterol/DNA and protein/DNA increase on the administration of testosterone. This would be expected since de novo synthesis of enzymes for cholesterol synthesis pathway would be required for an increase in cholesterol content.

Figure 3 shows the rate of synthesis of cholesterol, DNA and proteins at various periods of testosterone administration up to 14 days. The rates of synthesis of protein and cholesterol peak 2 days after testosterone injection, whereas DNA synthesis peaks after 4 days of treatment. The two peaks in protein and cholesterol synthesis after 2 days and again after 5 days might indicate the synthesis of structural components followed by synthesis of secretory components. After 5 days, synthesis of DNA, protein and cholesterol decreases and remains at a steady state for the remainder of the 14 days of testosterone treatment. Despite the fact that the rate of cholesterol synthesis per microgram of prostatic DNA reaches a steady state, the sharp increase in cholesterol content upon testosterone administration at day 12 can be accounted for by the increased prostate weight as seen in Fig. 1.

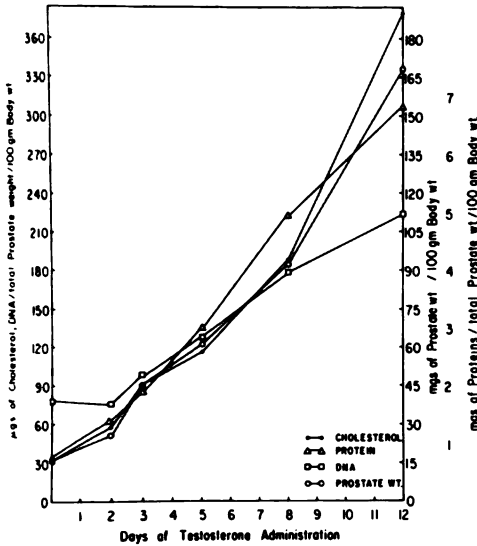


FIG. 1. The effect of testosterone administration to the castrated rats on prostate weight and the contents of cholesterol, DNA and protein in the ventral prostate. The mean values are obtained from groups of six rats.

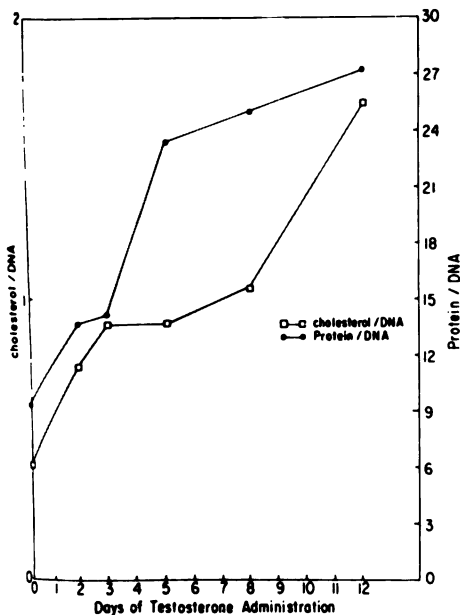


FIG. 2. The effect of testosterone administration to the castrated rats on the content ratios of cholesterol/DNA and protein/DNA in the ventral prostate. The mean values are obtained from groups of six rats.

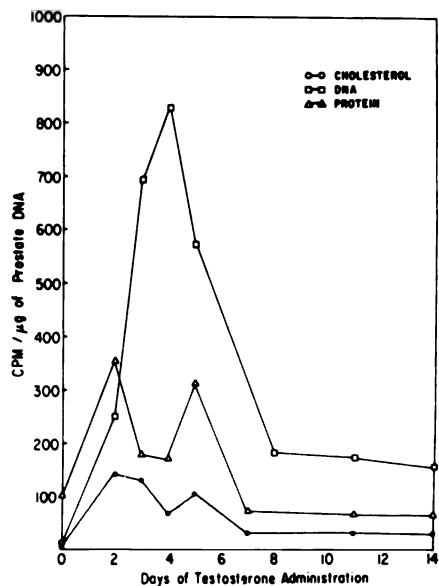


FIG. 3. The effect of testosterone administration to the castrated rats on the rates of synthesis of cholesterol, protein and DNA in the ventral prostate. The mean values are obtained from groups of six rats.

**Discussion.** Swyer (15) reported an increase in the cholesterol content of the adenomatous portion of enlarged human prostate glands as compared to normal glands. Braunstein (16) reported the presence of refractile and doubly refractile crystals as well as a positive Schultz reaction in the cytoplasm of human prostatic carcinoma cells indicative of a high content of cholesterol. Leav and Ling (17) reported the similar findings on tissues derived from neoplastic canine prostate gland.

Since the discovery that the hypocholesterolemic polyene macrolides (18) by the oral route decreased the size of the enlarged prostate glands of dogs (19) and hamsters (20), there has been increasing clinical evidence (21-27) that these drugs affect the symptoms of prostatism caused by benign prostatic hyperplasia. Other hypocholesterolemic agents such as cholestyramine (28), colestipol (20), simfibrate (29) and  $\beta$ -sitosterol (30) have now also been reported to affect the prostate gland. Considering that hypocholesterolemic drugs in general appear to affect the cholesterol-containing enlarged prostate gland and realizing the importance of cholesterol in this organ, it became necessary to study cholesterol metabolism in the prostate gland and its possible regulation by testosterone, a recognized mediator of other prostatic functions.

It is very evident from these current studies that testosterone is a major factor in the synthesis of cholesterol in the prostate gland. On testosterone administration to castrated rats the amount of cholesterol increases before an increase in DNA content. Liao *et al.* have shown that the RNA polymerase activity from the prostate of castrated rats is enhanced within a few hours of single injection of testosterone. This may mean that the initial increase in cholesterol content is more likely due to increased RNA and protein synthesis. Following the initial cholesterol curve there is an increase in DNA content and then another increase in cholesterol content.

The observed two different phases in the amount of prostate cholesterol, the first of parallel increase with protein from day 0 and the second of a sharp increase in cholesterol between day 8 and 12 can be explained on the basis of cholesterol having a dual function in the gland. In the first phase, it is likely that only structural or membrane cholesterol is

synthesized. After 8 days of testosterone administration when the gland approaches the normal state, since cholesterol is an important secretory product of the prostate gland, greater amounts of cholesterol-synthesizing enzymes might be produced as indicated by the large increase in protein content. This would be followed by the synthesis of a large amount of secretory cholesterol. Prostate weight rises in parallel with the amount of cholesterol.

The sharp increases in the synthesis of cholesterol, DNA and protein is followed by a sharp decrease on continuous testosterone administration. This may be due to a shift in testosterone metabolism in the prostate gland where testosterone may be converted to inactive or less active metabolites as compared to the conversion to a highly active metabolite such as dihydrotestosterone (31). This indicates that testosterone may be acting both as a positive and negative regulator of cholesterol synthesis in the prostate gland.

**Summary.** The absolute cholesterol content and rate of cholesterol synthesis was compared in rat ventral prostates obtained from adult normal and castrated rats. Cholesterol content and synthesis reduces to about 8-12% in the ventral prostate of castrated animals as compared to normal rats. Daily testosterone injections to castrated rats elicits a sharp increase in cholesterol content which correlates with an increase in prostate weight. The rate of cholesterol synthesis per microgram of prostatic DNA increases steeply 2 days after testosterone administration and then goes down and reaches a steady state after 5 days.

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# The Role of Cyclic AMP in CRF-Induced ACTH Secretion<sup>1</sup> (40272)

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Experiments in this (1) and other (2-4) laboratories have shown that cyclic-3',5'-adenosine monophosphate (cyclic AMP) and its derivatives stimulate the secretion of ACTH. Recently, we have reported that the stimulation of ACTH secretion by hypothalamic median eminence-corticotrophin releasing factor (HME-CRF) is associated with a concomitant increase in adenylate cyclase activity; however, cordycepin (3'-deoxyadenosine) at sufficient concentration to reduce adenylate cyclase activity to undetectable levels, reduces but does not abolish the HME-CRF induced secretion of the hormone (5). These data suggest that while cyclic AMP may be involved in CRF-stimulated ACTH secretion, the cyclic nucleotide may not act as an obligatory intermediate, but rather may act to potentiate secretion. The experiments described in the present communication were designed to provide further information on this hypothesis.

**Materials and methods.** The techniques used in the preparation and incubation of isolated pituitary cells have been described in detail elsewhere (6, 7). In brief, anterior pituitary glands were removed from male Sprague-Dawley rats which had been adrenalectomized 14-28 days prior to sacrifice, and were maintained after adrenalectomy on 0.9% saline drinking solution without steroid hormone replacement. Cells were dispersed from the glands by mechanical agitation in Krebs-Ringer bicarbonate (KRB) buffer containing 0.2% glucose and 0.25% trypsin. After dispersion, cells were collected by centrifugation and resuspended in KRB buffer containing 0.2% glucose and 0.5% bovine serum albumin (KRBGA), plus 0.1% lima bean trypsin inhibitor. Aliquots (0.9 ml) of cell suspension were incubated for various times together with appropriate combinations of HME-CRF, N<sup>6</sup>,O<sup>2</sup>-dibutyryl-cyclic AMP (DBC), corticosterone, or vehicle (controls).

At the end of the incubation period, cells were removed by centrifugation, and the incubation medium was acidified, appropriately diluted, and assayed for ACTH. In most cases, the samples were bioassayed according to the isolated adrenal cortex cell technique described by Sayers *et al.* (8), using synthetic ACTH 1-24 (Cortrosyn, Organon) as standard. In one experiment (employing concentrations of DBC greater than 1 mM, see Fig. 5), in order to circumvent the problem of direct DBC stimulation of steroidogenesis by isolated adrenal cells, pituitary cell incubation medium was assayed for ACTH by a radioimmunoassay (RIA) technique. Rabbit anti-human ACTH serum was purchased from Burroughs-Wellcome, and <sup>125</sup>I-ACTH 1-24 was obtained from Amersham. Samples or standards (ACTH 1-24, Cortrosyn, Organon) were incubated with immune serum in 0.1 M sodium phosphate (pH 7.4), for 20 hr (4°), at which time <sup>125</sup>I-ACTH was added and the incubation was continued for 6 additional hr. Un-bound <sup>125</sup>I-ACTH was adsorbed to charcoal, collected by centrifugation, and counted in a Packard auto-gamma spectrometer. The method appears valid as judged by several criteria: (a) both extracts of pituitary cells and samples of pituitary cell incubation media gave log dose-displacement curves parallel to synthetic ACTH 1-24; (b) a number of polypeptides, including ACTH 5-10, ACTH 5-13, and  $\alpha$ -MSH, showed no significant cross-reactivity; and (c) analysis of samples of pituitary cell extracts or incubation media by bioassay and RIA gave essentially identical values. In all experiments, the ACTH content of control incubates was determined and subtracted from that of incubates receiving test substance(s). In each experiment, data obtained from incubates receiving identical treatments were pooled, and means and standard errors of the means (SEM) were calculated. Statistical significance was assessed by means of Student's *t* test.

Extracts of rat hypothalamic median emi-

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nence tissue (HME-CRF) were prepared by homogenizing freshly excised ventral hypothalamic-median eminence tissue in 0.2 M acetic acid. Insoluble material was removed by centrifugation (20,000g, 15 min), and was reextracted twice with 0.2 M acetic acid. The extracts were combined and stored frozen. For use, a portion of the extract was adjusted to pH 7.0, appropriately diluted (with KRBGA) and added to the incubates in a volume of 0.1 ml. Doses of HME-CRF are expressed as tissue equivalents (i.e., fractions of an HME), which in these experiments had a wet weight of approximately 15 mg. Corticosterone (Sigma) in 0.9% saline plus 2.5% methanol, was added to appropriate incubates in a volume of 10  $\mu$ l. DBC (Sigma) was added to appropriate incubates in a volume of 0.1 ml of KRBGA.

**Results.** Both HME-CRF and DBC stimulate the secretion of ACTH by isolated pituitary cells (Fig. 1), and at the concentrations tested (0.2 HME/ml, 1 mM DBC) the ACTH secretory responses are nearly identical (150 pg/min/ $10^5$  cells). This concentration of DBC (1 mM) in the medium did not interfere in the subsequent steroidogenic bioassay for ACTH, as shown by the fact that addition of DBC at the end of the incubation period with HME-CRF does not significantly alter the response from that of HME-CRF alone. When pituitary cells are exposed to DBC throughout the exposure to HME-CRF, the rate of ACTH secretion is markedly enhanced. The rate of hormone secretion in the presence of HME-CRF plus DBC (575 pg/min/ $10^5$  cells) is almost twice that expected if the response to the two agents were simply additive. As shown in Fig. 2, the potentiating effect of DBC on HME-CRF induced ACTH secretion occurs without an obvious time-lag and persists throughout the duration of a 45 min incubation. The data in Fig. 3 indicate that the exposure of pituitary cells to DBC potentiates HME-CRF induced ACTH secretion, even if the cyclic nucleotide is removed prior to addition of HME-CRF. In these experiments cells were preincubated for 15 min in the presence or absence of DBC (1 mM) and then challenged with HME-CRF in the presence or absence of DBC (1 mM). HME-CRF induced ACTH secretion by cells exposed to DBC was more than twice that of

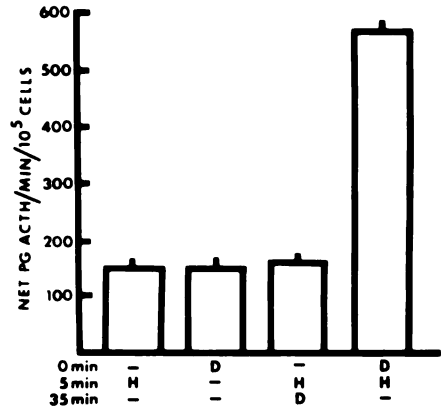


FIG. 1. Interaction of CRF and DBC on ACTH secretion. Isolated pituitary cells prepared from adrenalectomized rats were incubated for 35 min. Substances added, and their time of addition during this interval, are indicated below each bar: H, HME-CRF (.2 HME/ml); D, DBC (1 mM). Secretory rates are for the 30 min-period following the addition of HME-CRF; vertical lines represent combined SEM of pituitary and adrenal assays ( $N = 8$ ).

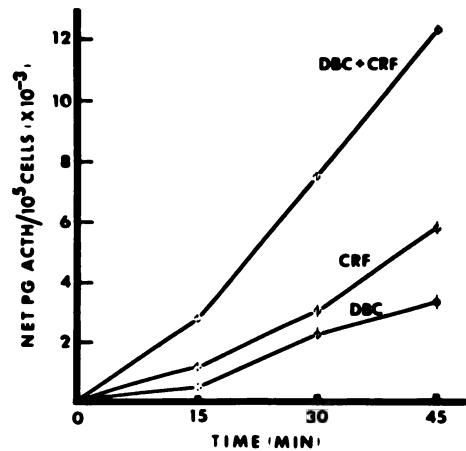


FIG. 2. Interaction of CRF and DBC on ACTH secretion; time course. Pituitary cells were incubated for indicated times in the presence of: DBC (1 mM), HME-CRF (.2 HME/ml), or DBC (1 mM) plus HME-CRF (.2 HME/ml). The ACTH content of control incubates ( $920 \pm 42$  pg/ $10^5$  cells, mean  $\pm$  SEM,  $N = 10$ ) did not change from 15 to 45 min, and has been subtracted from the experimental values presented. Vertical lines represent combined SEM of pituitary and adrenal assays ( $N = 4$ ).

cells which were not exposed to DBC, irrespective of whether the cyclic nucleotide was present during the preincubation only, the incubation with HME-CRF only, or both the



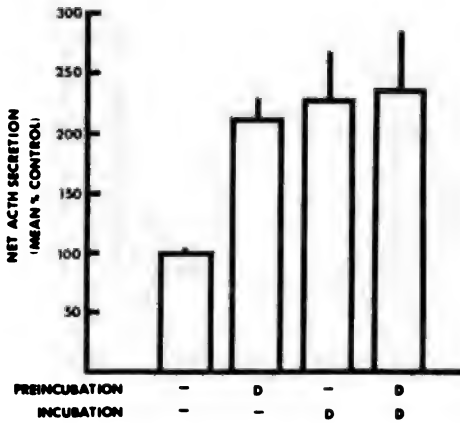


FIG. 3. Effect of time of addition of DBC on CRF induced ACTH secretion. Isolated pituitary cells were preincubated (15 min) in the presence or absence of DBC (1 mM); the cells were collected by centrifugation, washed with KRBGA, recollected by centrifugation, and resuspended in KRBGA. Aliquots of both types of cells were then incubated (30 min) with HME-CRF (.2 HME/ml) in the presence or absence of DBC (1 mM). The presence of DBC (D) during the preincubation and incubation periods is indicated beneath each bar. Data are expressed as the percentage of the secretory rate of cells which were not exposed to DBC (control); vertical lines represent SEM of the normalized secretory rates ( $N = 10$ ).

preincubation and the incubation.

The experiments described above demonstrate the interaction of submaximal doses of DBC and HME-CRF. In order to determine whether these secretagogues also interact at maximal dose levels, two experiments were performed. First, isolated pituitary cells were exposed to graded doses of HME-CRF in the presence or absence of DBC (Fig. 4). In the absence of DBC, maximum ACTH secretion is noted at a concentration of about 1.8 HME-CRF/ml. In the presence of DBC (1 mM), the secretory response to each dose of HME-CRF is increased more than twofold, even at maximum doses of HME-CRF. In the second experiment, isolated pituitary cells were exposed to graded doses of DBC in the presence or absence of HME-CRF (Fig. 5). In the absence of HME-CRF, maximum ACTH secretion is produced at a concentration of about 10 mM DBC. In the presence of HME-CRF (0.4 HME/ml), the secretory response is more than doubled at each dose of DBC, including the maximal doses.

Previous findings in our laboratory have

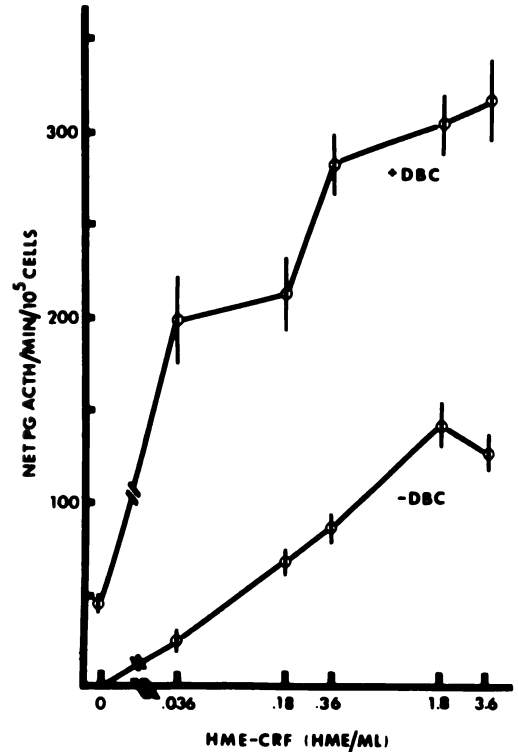


FIG. 4. Effect of DBC on ACTH secretion in response to graded doses of CRF. Isolated pituitary cells were incubated for 10 min in the presence or absence of DBC (1 mM); graded doses of HME-CRF were then added and the incubation was continued for an additional 30 min. Data presented are net pg ACTH secreted; vertical lines represent combined SEM of pituitary and adrenal assays ( $N = 8$ ).

shown that the secretion of ACTH by isolated pituitary cells in response to a variety of secretagogues, including DBC, is inhibited by corticosterone (9). We therefore carried out an experiment to determine if the potentiating effect of DBC on HME-CRF stimulated ACTH secretion is also inhibited by steroid. Pituitary cells were incubated for 30 min in the presence or absence of corticosterone (0.1  $\mu$ g/ml) and were then stimulated (for 30 additional min) with either HME-CRF (0.2 HME/ml), DBC (1 mM), or HME-CRF (0.2 HME/ml) plus DBC (1 mM). The data in Fig. 6 show, as expected, that in the absence of exposure to corticosterone, both HME-CRF and DBC stimulate the secretion of ACTH, and HME-CRF stimulated secretion is potentiated by DBC. When the cells are exposed to corticosterone, ACTH secretion

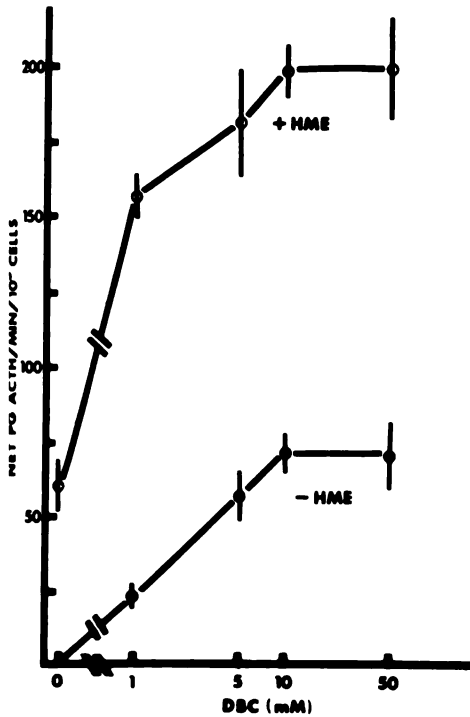


FIG. 5. Effect of HME-CRF on ACTH secretion in response to graded doses of DBC. Isolated pituitary cells were incubated for 30 min with various doses of DBC in the presence or absence of HME-CRF (0.4 HME/ml). ACTH was determined by RIA; data presented are net ACTH secreted. Vertical lines represent combined SEM of pituitary and radioimmuno assays ( $N = 6$ ).

nulated by either agent acting singly is early abolished; ACTH secretion stimulated by HME-CRF and DBC in combination is not markedly inhibited, but is still significantly greater than that induced by HME-CRF ( $P < .01$ ) or DBC ( $P < .01$ ), acting alone.

**Discussion.** Several lines of evidence suggest an involvement of cyclic AMP in the intracellular mechanisms which regulate ACTH secretion. Cyclic AMP and its derivatives have been found to stimulate the secretion of ACTH both *in vivo* and *in vitro* (1-4). Inhibitors of cyclic nucleotide phosphodiesterase stimulate the secretion of ACTH (4) or synergistically with other secretagogues of ACTH (2), presumably elevating the intracellular level of cyclic AMP. Recently, we observed that addition of HME-CRF to suspensions of isolated pituitary cells produces an increase in adenylate cyclase activ-

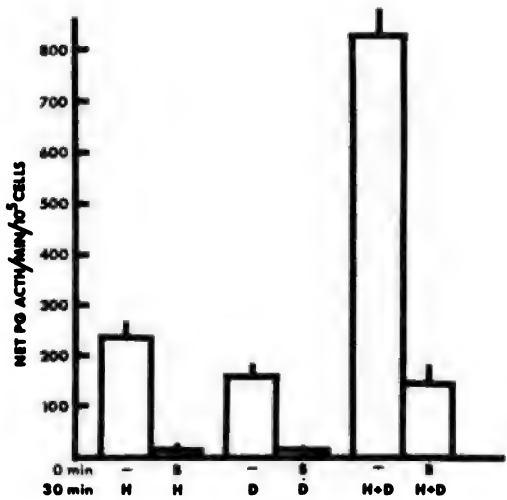


FIG. 6. Interaction of CRF and DBC on ACTH secretion; effect of corticosterone. Isolated pituitary cells were incubated for 60 min. Substances added, and their time of addition during this interval, are indicated below each bar; H, HME-CRF (0.2 HME/ml); D, DBC (1 mM); B, corticosterone (0.1  $\mu$ g/ml). Secretory rates are for the 30 min-period following the addition of HME-CRF; vertical lines represent combined SEM of pituitary and adrenal assays ( $N = 8$ ).

ity, concomitant with an increase in the rate of ACTH secretion (5). A stimulation of rat pituitary adenylate cyclase activity has also been reported in response to crude extracts of ovine hypothalamus (10) and vasopressin (4), an agent which is distinct from hypothalamic CRF but nevertheless stimulates the secretion of ACTH (11). These observations are all consistent with the notion that cyclic AMP is involved in the process which mediates ACTH secretion, but they provide no information as to the role of the cyclic nucleotide in this process. In this regard it is well to stress that although a large number of secretagogues of ACTH are known, no "authentic" hypothalamic CRF is yet available in pure form. Experiments employing crude extracts of hypothalamus (including those reported above) are limited in that responses observed may be the consequence of the interaction of several factors. Indeed, it is for this reason that little is known of the cellular and molecular processes which mediate ACTH secretion or the biochemical pathways by which these processes are regulated.

If the role of cyclic AMP in ACTH secretion is that of an obligatory "second messen-

ger", then it would be expected that an agent which inhibits adenylate cyclase activity would interfere with secretion of the hormone. Cordycepin has been found to be an inhibitor of adenylate cyclase activity in fat cell membranes (12) and guinea pig lung (13). Experiments in our laboratory (5) have shown that a dose of cordycepin sufficient to reduce adenylate cyclase activity to undetectable levels in isolated pituitary cells only partially reduces the rate of HME-CRF induced ACTH secretion. We interpreted these data to mean that while cyclic AMP may indeed be involved in CRF-stimulated ACTH secretion, the cyclic nucleotide may not act as an obligatory intermediate but rather may act to potentiate secretion. Sundberg *et al.* (14) have advanced a similar proposal with respect to the role of cyclic AMP in the secretion of several other adenohypophyseal hormones.

The data presented in this communication are consistent with this view. DBC potentiates HME-CRF induced ACTH secretion both at submaximal and maximal doses of HME-CRF (Fig. 4), and HME-CRF potentiates DBC induced ACTH secretion both at submaximal and maximal doses of DBC (Fig. 5). The mechanism of the interaction between HME-CRF and DBC is unknown. Potentiation occurs without an apparent lag period and persists for at least 30–45 min (Fig. 2). Significantly, pretreatment of isolated pituitary cells with DBC (followed by removal of the cyclic nucleotide prior to exposure to HME-CRF) potentiates the secretory response to HME-CRF to as great a degree as does exposure to HME-CRF in the presence of DBC (Fig. 3). This finding does not rule out the possibility that cyclic AMP has been sequestered within the cells during the pretreatment period, and subsequently potentiates hormone secretion during exposure to HME-CRF. Alternatively, this finding is consistent with the view that the potentiating effect following DBC pretreatment may represent a physical and/or chemical change in the cell which is exerted after the cyclic nucleotide has been removed.

The data in Fig. 6 dramatically illustrate the potent inhibitory effect of corticosterone on ACTH secretion. At concentrations (0.1  $\mu\text{g/ml}$ ) within the physiological range, the steroid markedly suppresses hormone secre-

tion in response to HME-CRF acting singly or in combination. These findings indicate that whatever the role of cyclic AMP in ACTH secretion, the stimulatory or inhibitory action of the steroid is distinct from the appearance of the cyclic nucleotide.

In conclusion, the data of the present communication support the hypothesis that cyclic AMP acts within corticotrophs to potentiate CRF-induced ACTH secretion. Our previous findings (5) indicate that an increased level of cyclic AMP is not sufficient for ACTH secretion to occur. Taken together, these data suggest that CRF has (at least) two actions on the corticotroph: (a) the initiation of the series of events which even includes secretory granule exocytosis, and (b) the regulation of cyclic AMP levels within the cell which then facilitates (through unknown mechanisms) the secretory process.

**Summary.** ACTH secretion by isolated pituitary cells is stimulated both by HME-CRF and DBC, and when given in combination the two secretagogues interact synergistically. Although the mechanism of this interaction is unknown, the potentiating effect is displayed without an apparent time lag and persists after removal of the cyclic nucleotide. Corticosterone inhibits ACTH secretion induced by HME-CRF and DBC, acting either alone or in combination. The implications of these findings are discussed.

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# Ketamine as an Anesthetic for Obtaining Plasma for Rat Prolactin Assays (40)

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Various procedures have been utilized to obtain blood from laboratory rats for assay of plasma prolactin levels. Because of the effect of stress and general anesthetic agents on plasma prolactin levels (1-4), blood sampling procedures which do not themselves affect plasma prolactin levels are limited. Dohler *et al.* (5) recently compared the influence of four methods of blood collection under three anesthetics, ether, chloroform and pentobarbital, to decapitation on the release of prolactin; in all instances they observed an increase in plasma prolactin ranging from 2- to 13-fold.

Lawson and Gala (4) reported that ketamine, which is a dissociative anesthetic, not a general anesthetic (6), produced no effect on plasma prolactin levels at 10, 30, 60 and 120 min after intraperitoneal (ip) or intra-arterial (i.a.) injection in ovariectomized rats with indwelling catheters. Lawson and Gala (7) subsequently reported that ketamine, 100 mg/kg, ip, also produced no change in plasma prolactin levels in catheterized, ovariectomized, estrogen-treated Sprague-Dawley rats, with sampling at 10, 30, 60 and 120 min after injection. However, ketamine, 50 mg/kg, i.a., significantly decreased plasma prolactin levels at 10, 60 and 120 min. They suggested that ketamine differed from other anesthetics in its effects on prolactin secretion because it induced only stage II anesthesia.

We were interested in determining what effects if any, ketamine had on plasma prolactin levels in male rats. Since ketamine has been shown to inhibit both dopamine and serotonin uptake (8, 9), two neurotransmitters which have a profound effect on rat prolactin secretion (10, 11), it was of further interest to see if ketamine affected baseline prolactin levels or the reserpine,  $\alpha$ -methylparatyrosine (AMPT)- or 5-hydroxytryptophan (5-HTP)-induced increase in prolactin secretion. Drugs which inhibit 5-HT uptake will promote the

increase in prolactin produced by (12).

**Methods.** Male Sprague-Dawley (Sprague-Dawley, Inc., Madison, WI) weighing 200-225 g were housed for at least 1 week in a temperature-controlled (25°) and controlled (6 AM-8 PM light period) room. They received food and water *ad libitum*. Ten groups of five rats each had catheters placed in the right jugular vein under pentobarbital anesthesia (13). The catheters were kept patent with flushing with saline on alternate days. These rats were handled frequently and accustomed to the procedure of blood withdrawal of 0.3 ml blood. These rats received no anesthesia at the time of blood withdrawal. Another ten groups of five rats each were administered ketamine, 100 mg/kg, ip immediately after they became unresponsive to toe pinch (usually 2-3 min). Blood was withdrawn from the inferior vena cava. Finally, ten groups of five rats each were rapidly decapitated.

Reserpine, 5 mg/kg, ip, was given 15 min or 3 hr 30 min before ketamine, 100 mg/kg, ip, or saline. Rats were sacrificed 4 hr after reserpine. AMPT, 100 mg/kg, was given 15 min before ketamine, 100 mg/kg, ip and rats were sacrificed 4 hr later. To determine the effects of ketamine on 5-HTP-induced increases in plasma prolactin, ketamine, 25, 50, and 100 mg/kg, was given 30 min before 5-HTP, 30 mg/kg. For comparison purposes, one group was pretreated with fluoxetine (Lilly 10360), a known 5-HT reuptake blocker (14), and another group was pretreated with saline, followed by 5-HTP, as described for the ketamine-treated rats.

Following sacrifice, plasma samples were frozen and assayed later for prolactin by modification of a double antibody radioimmunoassay originally developed for prolactin assay (15). Prolactin levels

l in terms of NIAMDD-rat prolactin. All samples utilized in this report were d together. The sensitivity of the assay ig/ml. The intra-assay variation is less %.

etermine if there was a difference in prolactin levels between types of sacrifice means for the 10 groups of each type ompared with a one way analysis of ce (ANOVA). To examine for differ- in variance within each of the three ents, a completely randomized hierar- analysis of variance was performed he effect of drugs on the increase in tin produced by 5-HTP was deter- by an ANOVA.

amine HCl was generously supplied by Davis-Warner-Chilcott, Inc., Ann Ar- lich. Alpha-methylparatyrosine meth- and 5-hydroxytryptophan methylester urchased from Sigma, Inc., St. Louis, Reserpine was obtained from Ciba- Corp., Summit, NJ. Fluoxetine was a Eli Lilly, Co., Indianapolis, IN. All oses refer to the salt form.

lts. Prolactin levels for the various of sacrifice are summarized in Table I. edian, range and coefficient of varia- v.) were calculated utilizing the mean r each group of five rats.

results of an ANOVA indicated there significant difference between any of

the three methods of blood collection. How- ever, five of the ten groups of catheter sam- ples had mean levels that exceeded the high- est mean of the ketamine groups (10.2 ng/ml). Only one of the decapitated groups had a mean plasma prolactin which exceeded 10.2 ng/ml. The ketamine-treated group had the lowest prolactin levels and the smallest coefficient of variation of the three types of treatment.

Ketamine did not significantly affect the increase in plasma prolactin levels produced by reserpine or AMPT (Table II).

5-Hydroxytryptophan, 30 mg/kg, or fluox- etine, 10 mg/kg, did not increase plasma prolactin levels (Table III). Fluoxetine, to- gether with this dose of 5-HTP, produced a very significant increase in plasma prolactin. However, none of the three doses of ketam- ine, plus 5-HTP had any effect on plasma prolactin levels. Fluoxetine plus ketamine, 100 mg/kg, also did not augment plasma prolactin.

*Discussion.* The results of the studies in untreated male rats strongly indicate that an- esthesia with ketamine does not affect plasma prolactin levels. Blood obtained from the in- ferior vena cava within 3 min of administra- tion of ketamine has levels of prolactin not significantly different from that obtained from decapitated rats or from rats with in- dwelling venous catheters. The latter method

TABLE I. RAT PLASMA PROLACTIN LEVELS FOLLOWING KETAMINE, GUILLOTINING AND FROM INDWELLING CATHETERS.

Group	N	Mean $\pm$ SEM	Median	Range*	Mean coefficient of variation (%)
mine	5 rats, $\times 10$	6.0 $\pm$ 0.8	6.3	1.9-10.2	58.0
pitiation	5 rats, $\times 10$	6.4 $\pm$ 1.2	6.8	1.8-13.5	72.0
eter	5 rats, $\times 10$	9.4 $\pm$ 1.9	8.9	2.2-19.8	63.4

is of each group of 5.

TABLE II. EFFECT OF KETAMINE ON PLASMA PROLACTIN LEVELS FOLLOWING RESERPINE OR AMPT.

		Plasma prolactin (ng/ml)*		
	Dose (mg/kg)	Saline	Ketamine	p
reserpine (A)	5	18.5 $\pm$ 3.7	25.0 $\pm$ 3.1	NS
reserpine (B)	5	21.7 $\pm$ 2.7	21.3 $\pm$ 1.7	NS
AMPT	100	15.4 $\pm$ 4.6	15.2 $\pm$ 3.9	NS

in  $\pm$  SEM Ketamine, 100 mg/kg ip or saline was given 3 hr 55 min (A) or 3 hr 30 min (B) following : and 15 min following AMPT. Rats were sacrificed by decapitation 5 min (A) or 30 min (B) after ketamine serpine-pretreated rats, and 15 min after ketamine in the AMPT-pretreated rats. All groups consisted of 5

TABLE III. EFFECT OF KETAMINE AND FLUOXETINE ON INCREASE IN PROLACTIN PRODUCED BY 5-HTP.

Pretreatment	Dose (mg/kg)	Treatment	Dose (mg/kg)	Plasma prolactin* (ng/ml)
Saline	—	Saline	—	6.4 ± 1.5
Saline	—	5-HTP	30	8.4 ± 1.5
Fluoxetine	10	Saline	—	7.5 ± 1.4
Fluoxetine	10	5-HTP	30	38.7 ± 4.6
Fluoxetine	10	Ketamine	100	6.4 ± 1.3
Ketamine	25	5-HTP	30	6.7 ± 2.3
Ketamine	50	5-HTP	30	8.6 ± 1.3
Ketamine	100	5-HTP	30	7.0 ± 1.4

\* Mean ± SEM. The first injection was given 60 min before the second injection. Groups of five rats were sacrificed by decapitation 15 min after saline or 5-HTP.

of blood sampling tended to produce the highest levels and the greatest variance within a given group of 5 rats, the usual size of our control groups. These results indicate that where a single blood sample is required from a given male rat, ketamine anesthesia is acceptable. For studies in which anesthetized rats might be desirable, ketamine is clearly preferable to other anesthetics which themselves affect prolactin secretion. The reported ability of ketamine, 50 mg/kg, i.a., to lower prolactin levels, in ovariectomized estrogen-treated rats (7), if confirmed, would indicate that ketamine might affect the estrogen-stimulated prolactin secretion process and thus be less suitable for use in studies with female rats than it appears to be for male rats. The lack of effect of ketamine on prolactin secretion further documents the difference between the anesthesia produced by this agent and classical general anesthetics.

The inability of ketamine to reverse the increase in plasma prolactin levels produced by reserpine or AMPT is strong evidence that ketamine does not have direct dopamine agonist effects *in vivo* at the pituitary dopamine receptors which regulate prolactin secretion. Direct dopamine agonists such as apomorphine, bromocriptine or lysergic acid diethylamide readily reverse the increase in prolactin produced by reserpine or AMPT (17, 18 and unpublished data from this laboratory). Similarly, the inability to reverse the reserpine or AMPT-induced increase in prolactin indicates ketamine differs significantly from d-amphetamine, which has been shown to reverse the increase in prolactin secretion pro-

duced by reserpine or AMPT (19), presumably by increasing the release of dopamine from tubero-infundibular dopamine neurons or blocking its uptake. Previous studies of the effect of ketamine on dopaminergic mechanisms have been *in vitro* and have dealt with the nigro-striatal dopaminergic pathway. These differences may account for the differences between the results of those studies and this one.

The ability of fluoxetine but not ketamine to potentiate the effects of a subthreshold dose of 5-HTP on prolactin secretion indicates that ketamine is not an effective inhibitor of serotonin reuptake *in vivo* at those neurons which release the serotonin that potentiates prolactin secretion. These are believed to be the median raphe serotonergic neurons (20). However, an effect of ketamine on uptake of serotonin by other serotonergic neurons is not excluded.

The lack of effect of ketamine on the reserpine-, AMPT- and 5-HTP-induced increase in prolactin secretion indicates the suitability of ketamine for anesthesia in studies of the effect of dopaminergic and serotonergic drugs on prolactin secretion.

**Summary.** Mean plasma prolactin levels obtained from male rats following anesthesia with ketamine, decapitation or via indwelling venous catheters were not significantly different although a larger variance was found in the samples obtained via catheters. Ketamine, at anesthetic doses, did not affect the increases in prolactin produced by reserpine or  $\alpha$ -methylparatyrosine. Ketamine, at various doses, did not potentiate the effect of subthreshold doses of 5-hydroxytryptophan on prolactin secretion. Thus, ketamine would appear to be a suitable anesthetic for use in studies of prolactin secretion in male rats. Further studies in female rats are required.

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## Pyrazinoic Acid and Urate Transport in the Rat (40274)

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The decrease in urinary excretion of urate following the administration of pyrazinamide or its active metabolite, pyrazinoic acid (PZA), has been extensively utilized as a pharmacologic aid in dissecting out the contribution of secreted urate to the urinary excretion of uric acid (1, 2). As originally proposed, the use of the "Pyrazinamide Suppression Test" was based upon the assumptions that this compound was a specific and perhaps complete inhibitor of urate secretion and was without effect on the urate reabsorptive processes (3, 4). Indirect evidence has been presented, however, that neither of these assumptions is totally valid (5-8). Published studies on the separate effects of PZA on urate reabsorption and secretion, however, have been limited and somewhat conflicting (8-11). The current studies were designed to examine the effect of PZA, in varying dosages, on net urate transport and on the urate reabsorptive and secretory mechanisms in the rat.

**Methods.** Male Sprague-Dawley rats with free access to food and water until the time of study were used in all experiments. Anesthesia was induced with Inactin (Promonta, Hamburg, Germany), 0.5-0.6 mM/kg body wt injected intraperitoneally. After a tracheostomy, the right and left jugular veins were cannulated and the urinary bladder catheterized. In the clearance experiments, the left femoral artery was cannulated for collection of blood samples. In the microinjection and precession studies, the left kidney was prepared for micropuncture as previously described (12, 13). The ureter of the left kidney was catheterized with PE-50 tubing to permit separate urine collections from each kidney. Only animals in which the urine flow rate of the left kidney was at least 85% of that from the contralateral kidney were included for study. In all animals, surgical losses of fluid were replaced with a volume of isotonic saline equal to 1% of body wt. Body temperature

was maintained at 37°. Pyrazinoic acid dissolved in a solution of sodium hydroxide (0.1 M); the pH was then adjusted to 7.4 with either hydrochloric acid or sodium bicarbonate. In all control periods, the diluent was infused to control for the effect, if any, of diluent infusion.

**Clearance studies.** Clearance studies were performed in diuretic rats receiving 5% mannitol in isotonic saline at a rate of 12.0 ml/hr so as to reproduce the protocol of the microinjection studies which require high urine flow rates. A priming dose of 50  $\mu$ Ci of [*met*-<sup>3</sup>H] inulin in one ml of isotonic saline was infused followed by a sustaining infusion of isotonic saline containing 25  $\mu$ Ci/ml of [*oxy*-<sup>3</sup>H] inulin at a rate of 1.2 ml/hr. After a 90-min equilibration period, two 20-min urine collections were obtained. 1.5 ml of arterial blood was obtained at the midpoint of each clearance period and was replaced with the same volume of blood from a donor rat.

After collection of samples in the clearance periods, pyrazinoic acid in a dose of 0.40, 0.80, or 1.6 mM/kg body wt (50, 100, or 200 mg/kg body wt respectively) was injected intravenously as a bolus followed by the dose infused per hour. After a 90-min equilibration period, two or three additional clearance periods were obtained. In order to control for possible changes in renal function over the time course of these experiments, five rats were studied under the same protocol but received no infusion of drug. At the conclusion of all experiments, the kidneys were removed, stripped of perirenal fat and capsule, and weighed in a Mettler analytic balance (Mettler Instrument Corp., Princeton, N.J.).

**Microinjection studies.** Microinjection studies were performed in animals receiving mannitol in isotonic saline at a rate of 12.0 ml/hr. Inulin was not infused systemically. After preparation for study, separate groups of animals received either diluent infusion or a bolus infusion of pyrazinoic acid of

1.6 mM/kg body wt followed by the dose per hour. An equilibration period of 90 min was permitted to elapse before microinjections. Intratubular microinjections were performed with a solution containing [2-<sup>14</sup>C]urate (50  $\mu$ Ci/ml) and [ $\gamma$ -<sup>3</sup>H]inulin (100  $\mu$ Ci/ml) adjusted to pH 7.4 with a solution of NaHCO<sub>3</sub> (0.357 M). The concentration of uric acid in the solution was 0.24 mM/liter. Triplicate aliquots of 12–20 nl were prepared, one of which was utilized for the microinjection; the other two counted directly for total radioactivity. Microinjections were performed into early or late proximal tubular segments at a 60–90 sec interval and total urine samples obtained sequentially from both right and left kidneys. The procedures for microinjection, localization of microinjection, and the calculations of the recovery rates were identical to those of Kramp, Lassiter, and Stetschalk (8) and have been described in detail from this laboratory previously (12).

**Droplet studies.** Animals were prepared as in microinjection studies except that 5% of the volume in isotonic saline was infused at a rate sufficient to increase the urine flow rate to 150  $\mu$ l/min per kidney. 100 nanoliters of [2-<sup>14</sup>C]urate and [methoxy-<sup>3</sup>H]inulin were placed upon the surface of the kidney as a droplet and urine collected fractionally in 15–30 sec aliquots from both right and left kidneys. A sample of the droplet was counted directly with each aliquot to determine the ratio of <sup>14</sup>C counts to <sup>3</sup>H counts. Droplet studies were obtained in control animals and in animals infused with PZA in doses of 0.40, 0.80, or 1.6 mM/kg body wt/hr as previously indicated. A sample was made to quantitate total radioactivity.

**Analytical methods.** Radioactivity of blood, urine, and microinjection and droplet samples was determined in Biofluor (New England Nuclear Corp., Boston, MA) in a Packard-Carb liquid scintillation counter (Model 4600, Packard Instruments Co., Downers Grove, IL) with appropriate corrections for <sup>14</sup>C appearing in the <sup>3</sup>H channel. Counts were converted to disintegrations per minute after correction for quench, crossover, and efficiency of counting each isotope. The

urate concentrations of the serum and urine were determined by a uricase method using the polarographic sensor in a glucose analyzer (Beckman Instruments, Fullerton, CA) as previously described (12). The clearances of inulin ( $C_{\text{inulin}}$ ) and urate ( $C_{\text{urate}}$ ) are expressed as  $\mu$ l/min/g kidney wt and are calculated from standard formulae.

All data are expressed as the mean  $\pm$  SE of the mean. *P* values were calculated by the Fisher *t* test or the Student *t* test where appropriate.

**Results. Clearance studies (Table I).** Following the infusion of PZA in a dose of 0.40 mM/kg body wt/hr, there was no change in the glomerular filtration rate, plasma urate concentration or in the clearance of urate. The fractional excretion of urate, therefore, was unchanged and averaged  $21.0 \pm 1.3$  and  $24.0 \pm 2.3\%$  (*P* = NS) in control and experimental periods respectively. By contrast, the infusion of PZA in a dose of 0.80 mM/kg body wt/hr resulted in significant decreases in urate clearance from  $276.0 \pm 25.1$  to  $210.7 \pm 20.6$   $\mu$ l/min/g kidney wt (*P* < 0.005) and in the fractional excretion of urate from  $24.4 \pm 2.6$  to  $19.4 \pm 2.4\%$  (*P* < 0.01). The plasma concentration of urate increased from  $58.3 \pm 4.2$  to  $86.8 \pm 5.4$   $\mu$ M/liter (*P* < 0.001). The infusion of PZA in a dose of 1.6 mM/kg body wt/hr resulted in no change in plasma urate concentration, the glomerular filtration rate, or the clearance of urate.

In order to control for the time course of these experiments, animals studied in identical fashion but not receiving an infusion of PZA, had no significant change in the glomerular filtration rate, the plasma urate concentration, or the clearance of urate.

**Microinjection studies (Fig. 1).** To assess the effects of varying dosages of PZA on the urate reabsorption process and to localize the nephron site of altered reabsorption, intratubular microinjections were performed into early or late portions of the proximal tubule. Only samples in which inulin recoveries were 95% or greater were included for analysis. Delayed recoveries ranged from 0 to 6% with no significant differences between the groups of animals. Accordingly, the results are expressed as total urate recoveries and are summarized on Fig. 1. Recoveries from early proximal tubule sites averaged  $73 \pm 2\%$  in

TABLE I. THE EFFECTS OF PZA ON THE CLEARANCE OF URIC ACID.<sup>a</sup>

Dose of PZA infused	C <sub>creatinine</sub> $\mu\text{l/min/g kw}$		Serum Uric Acid $\mu\text{M/liter}$		C <sub>uric acid</sub> $\mu\text{l/min/g kw}$		FE <sub>uric acid</sub> (%)	
	C	E	C	E	C	E	C	E
No PZA (n = 5)	1016 $\pm$ 89.3	953 $\pm$ 56.4	67.8 $\pm$ 6.5	70.2 $\pm$ 5.9	203.0 $\pm$ 25.0	217.2 $\pm$ 22.7	19.2 $\pm$ 1.7	23.1 $\pm$ 2.8
P		NS		NS		NS		NS
0.40 mM/kg/hr (n = 8)	1069 $\pm$ 54.7	1007 $\pm$ 65.2	70.8 $\pm$ 3.0	67.2 $\pm$ 3.0	221.6 $\pm$ 12.2	237.5 $\pm$ 24.7	21.0 $\pm$ 1.3	24.0 $\pm$ 2.3
P		NS		NS		NS		NS
0.80 mM/kg/hr (n = 6)	1169 $\pm$ 75.8	1175 $\pm$ 92.8	58.3 $\pm$ 4.2	86.8 $\pm$ 5.4	276.0 $\pm$ 25.1	210.7 $\pm$ 20.6	24.4 $\pm$ 2.6	19.4 $\pm$ 2.4
P		NS		<0.001		<0.005		<0.01
1.60 mM/kg/hr (n = 9)	967 $\pm$ 36.4	1054 $\pm$ 62.9	61.9 $\pm$ 4.8	68.4 $\pm$ 5.9	230.6 $\pm$ 25.7	260.6 $\pm$ 27.4	24.6 $\pm$ 3.5	25.5 $\pm$ 3.0
P		NS		NS		NS		NS

<sup>a</sup> Values expressed as mean  $\pm$  SEM. FE<sub>uric acid</sub> = fractional excretion of uric acid; C = control periods; E = experimental periods; NS = not significant; (n) = number of animals studied.

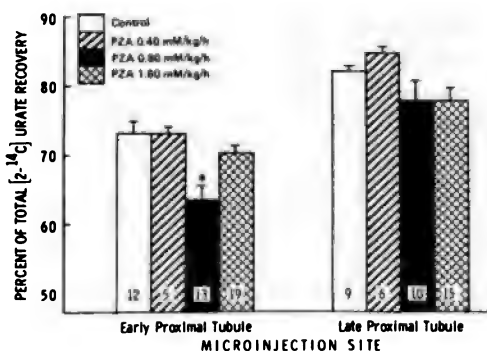


FIG. 1. Per cent of total  $[2-^{14}\text{C}]$ -urate recovered following microinjections in early and late proximal tubule sites. \*  $P < 0.01$ .

controls. Following infusion of PZA in doses of 0.40, 0.80, or 1.6 mM/kg body wt/hr, recoveries from early proximal tubule sites were  $73 \pm 1$ ,  $64 \pm 2$ , and  $71 \pm 1\%$  respectively. The urate recoveries after infusion of 0.80 mM/kg body wt/hr PZA ( $64 \pm 2\%$ ) were significantly lower than those obtained in controls and in animals infused with PZA in doses of either 0.40 or 1.6 mM/kg body wt/hr. There were no differences in urate recoveries following microinjections in late proximal tubule sites between any of the groups of animals.

**Droplet studies (Table II).** Urate secretion was considered to be present when the ratio of  $[2-^{14}\text{C}]$ urate to  $[\text{methoxy-}^3\text{H}]$ inulin in the first urine sample to contain inulin divided by the ratio of  $^{14}\text{C}/^3\text{H}$  in the droplet solution was greater than one. In control animals not receiving PZA, the  $^{14}\text{C}/^3\text{H}$  urine-to-droplet ratio of counts averaged  $1.79 \pm 0.10$  in the experimental left kidney and  $0.79 \pm 0.07$  in the contralateral kidney. The infusion of PZA in a dose of 0.40 mM/kg body wt/hr resulted in an 11% decrease in the ratio of counts in

the left kidney ( $P < 0.05$ ) and no significant change in the right kidney. Compared to controls, PZA in a dose of 0.80 mM/kg body wt/hr resulted in a significant decrease in the ratio of counts from  $1.79 \pm 0.04$  to  $1.19 \pm 0.12$  ( $P < 0.05$ ) and  $0.79 \pm 0.04$  to  $0.57 \pm 0.07$  ( $P < 0.05$ ) in the left and right kidneys respectively. The largest dose of PZA tested (1.6 mM/kg body wt/hr) resulted in a 38% decrease in the ratio of counts in the experimental left kidney ( $P < 0.05$ ) but no significant change in the right kidney.

**Discussion.** The presence of active mechanisms for the bidirectional transport of urate by renal tubular cells has made it difficult to assess the individual contribution of urate reabsorption or secretion to the urinary excretion of urate by classical clearance techniques. Pyrazinamide or its active metabolite, pyrazinoic acid (PZA), has been extensively utilized in man and in the intact animal as a pharmacologic aid in assessing the magnitude of each of these transport processes (1-3). The use of PZA in such studies was based upon the observation that, following its administration, the urinary excretion of urate was markedly reduced, an effect ascribed to an inhibition of urate secretion (1-3). More recently, doubt has been cast upon the results of studies utilizing the PZA-induced decrease of urate excretion as an index of urate secretion (4-6).

Prior studies from this and other laboratories have attempted to estimate urate reabsorption and urate secretion utilizing intratubular microinjection and droplet precession techniques, respectively. The rationale behind these techniques has been previously discussed (8, 9, 12-15). PZA in a dose of 0.40 mM/kg body wt/hr did not affect the fractional excretion of urate or the rate of urate

TABLE II. PRECESSION DROPLET STUDIES."

	Left kidney				Right kidney			
	C	E	% Change	P	C	E	% Change	P
(n = 4)	1.79 ± 0.10	1.79 ± 0.02	0	N.S.	0.79 ± 0.07	0.79 ± 0.06	0	N.S.
/liter (n = 4)	1.97 ± 0.07	1.76 ± 0.06	-11%	<0.05	0.79 ± 0.05	0.76 ± 0.04	-4%	N.S.
/liter (n = 4)	1.79 ± 0.14	1.19 ± 0.12	-34%	<0.05	0.79 ± 0.04	0.57 ± 0.07	-28%	<0.05
/liter (n = 4)	1.86 ± 0.12	1.16 ± 0.09	-38%	<0.05	0.69 ± 0.02	0.67 ± 0.03	-3%	N.S.

ntrol. E = experimental. Values (mean ± SEM) represent the  $^{14}\text{C}/^1\text{H}$  urine/droplet ratios of counts in the first urine sample to contain inulin.

ies following intratubular microinjection did, however, have a small but measurable effect on urate secretion as assessed by droplet studies. This apparent discrepancy may indicate that either the degree of inhibition of secretion was not physiologically significant, or that it could not be detected by clearance or microinjection techniques. In contrast to the 0.40 mM dose, PZA in a dose of 0.80 mM/kg body wt/hr resulted in a decrease in the fractional excretion of urate. This decrease in urate excretion could be the result of either inhibition of urate secretion, inhibition of urate reabsorption, or a combination of the two. The results of the precession droplet studies confirm that PZA inhibits urate secretion, the degree of inhibition being more pronounced with the 0.80 mM dose than with the 0.40 mM dose. The intratubular microinjection studies indicate that urate absorption is not affected. The mechanism by which PZA enhances urate absorption is unknown, and several possibilities might be considered. On the other hand, the decrease in urate recoveries following microinjection represent a direct pharmacologic effect on the reabsorption of urate from the renal tubule. This suggestion has previously been proposed from clearance experiments (6, 7). On the other hand, the decreased renal recovery of urate following intratubular microinjection in animals receiving a dose of 0.80 mM may be due to an inhibition of peritubular uptake of urate or to urate secretion alone. It is possible that inhibition of urate uptake at the antiluminal border of the renal tubular cells reduces the luminal concentration of urate, thereby creating a more favorable lumen-to-cell gradient for reabsorption. Moreover, inhibition of secretion of urate into the tubular lumen would increase the specific activity of the microinjected [ $^{14}\text{C}$ ]urate. Prior studies from this laboratory have indicated that reducing the specific activity of isotopically labeled urate in the tubular lumen does not affect the fractional

rate of [ $^{14}\text{C}$ ]urate absorption (13). The effect of increasing the specific activity, however, has not been examined directly and, thus, the expected changes in specific activity of [ $^{14}\text{C}$ ]urate microinjected into the tubular lumen can not be excluded as a possible mechanism, at the present time. The current studies do not permit us to differentiate between a direct pharmacologic effect of PZA on the urate absorptive mechanisms and an effect of PZA solely on the secretory process with a secondary change in the absorptive process, but the results of studies using PZA in a dose of 1.6 mM/kg body wt/hr suggest that the latter is the more likely explanation, namely that PZA in a dose of 0.80 mM enhances urate absorption, primarily by inhibition of the secretory process. With the largest dose of PZA tested, fractional urate excretion and fractional urate recoveries following microinjections were similar to control values. This dose of PZA also significantly inhibited urate secretion. It seems likely that PZA, 1.6 mM/kg body wt/hr, not only inhibits secretion, but also inhibits reabsorption and, at this dose, secretion and reabsorption were inhibited to an equal extent. When viewed from this perspective, PZA appears to inhibit both urate secretion and urate reabsorption, and the inhibition of these processes is dose-dependent, but not necessarily of equal sensitivity. It was unfortunate that, due to an unacceptably high death rate of the animals, higher doses of PZA could not be examined.

Three previously published studies on the effect of pyrazinamide or PZA on the renal handling of urate in the rat bear directly on the results in the present study. A significant decrease in urate reabsorption has been reported by Kramp *et al.* when single bolus doses of PZA of either 10, 50, or 100 mg/kg body wt/hr were infused (8). The differences between their results and those of the current study can not be readily reconciled. In a series

of clearance studies, Boudry observed a small antiuricosuric effect of PZA, an effect which became more pronounced when the plasma urate concentration was increased (16). In a more recent study by Abramson and Levitt, there was an increase in net reabsorption by the end of the proximal tubule following PZA administration, a result ascribed to inhibition of secretion (11). Also observed in that study was a significant reabsorptive flux of urate in the loop of Henle following PZA infusion. In the current study, recoveries from late proximal tubule sites were lower than controls following PZA administration, but the changes were not statistically significant. Thus, we can not confirm or deny, at this time, an effect of PZA in nephron sites beyond the proximal convoluted tubule.

The use of pyrazinoic acid depression of urate excretion as an index of urate secretion has been based upon the assumptions that PZA inhibits urate secretion and is without effect on urate reabsorption. The results of the present studies confirm that PZA inhibits urate secretion, and thereby may secondarily enhance urate absorption. In high doses, however, PZA has the additional effect of inhibiting urate reabsorption. To the degree that PZA may affect both urate secretion and reabsorption, any conclusions derived from the use of PZA as to the magnitude of the contribution of secreted urate to the urinary excretion of urate can not be considered quantitative.

**Summary.** These results indicate that urate secretion is inhibited by PZA and that the degree of inhibition is dose dependent. In the highest dose tested (1.6 mM/kg body wt/hr), PZA not only inhibits secretion but also inhibits urate absorption. Thus, PZA appears to inhibit both urate secretion and reabsorption. The inhibition of these processes is dose dependent but not necessarily of equal sensitivity.

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# Increased Antiviral Effect of Phosphonoacetic Acid on the Poikilothermic Herpesvirus of Channel Catfish Disease (40275)

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Recently, a characteristic sensitivity to the phosphonoacetic acid (PAA) has been demonstrated for representative herpesviruses of mammalian (1-8) and avian (9) species. In each reported system virus expression has been significantly inhibited in the presence of 100 µg/ml or less concentration of PAA. This mode of inhibition has been defined to be interference of virus-coded DNA polymerase activity (10, 11) and due to this specificity the therapeutic aspects of this drug in mammalian herpesvirus systems currently appear quite promising (12).

We have investigated PAA in a cold-blooded (poikilothermic) herpesvirus system to evaluate the eventual possibility of disease control. Channel catfish herpesvirus (CCV) is the etiologic agent (13, 14) of an economically devastating disease well known to the commercial aquaculture industry (15). We found that expression in cell culture to be inhibited by PAA. However, 10-20 times the drug concentration was required compared to that amount necessary to inhibit warm-blooded (homeothermic) herpesvirus systems.

**Materials and methods.** *Viruses, cell, receptors.* Channel catfish virus strain Auburn (A) originally received from Dr. John Harshbarger (Auburn University, Auburn, AL) was maintained at 25° in a continuous cell line of northern bullhead catfish (BB) cells. Channel catfish virus strain Homestead (CCV<sub>H</sub>) was isolated from an epizootic of channel catfish disease which occurred in South Florida (Koment, unpublished). This strain differs from the Auburn strain in its plaque morphology and complete lack of syncytial celling and cytopathic effects in BB cell culture.

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BB cells were grown at 25° in 75cm<sup>2</sup> plastic tissue culture flasks under Eagle's medium supplemented with 10% fetal calf serum, 0.075% sodium bicarbonate, 100 units/ml of penicillin and 100 µg/ml streptomycin.

Stocks of herpes simplex viruses (HSV) type 1 (HSV-1) strain 2bb and herpes simplex virus type 2 (HSV-2) strain 196 were prepared in human embryo lung cell cultures (Flow 2000). Primary rabbit kidney (pRK) and baby hamster kidney (BHK) cells were cultured at 37° under the same growth medium as described above for BB cell cultures.

Disodium phosphonoacetate was obtained from Abbott Laboratories (Chicago, IL). Dilutions were prepared in either maintenance medium (Eagle's medium supplemented with 2% fetal calf serum, 0.075% sodium bicarbonate, 100 units/ml of penicillin and 100 µg/ml of streptomycin) or overlay medium (Eagle's medium supplemented with 0.5% methylcellulose, 5% fetal calf serum, 0.23% sodium bicarbonate, 100 units/ml of penicillin and 100 µg/ml of streptomycin).

**Virus plaque assay, plaque reduction by PAA.** A standard virus plaque assay was developed for channel catfish virus in BB cells under Eagle's medium containing 0.5% methylcellulose. This was with modifications based on procedures previously described for the *in vitro* assay of herpes simplex virus (16). Briefly, tenfold serial dilutions of CCV were prepared and inoculated onto confluent monolayers of BB cells in 35 mm plastic dishes. After 1 hr. incubation at 25° to allow virus adsorption, 2 ml of overlay medium was added per dish and cultures incubated at 25° in a 5% CO<sub>2</sub> atmosphere. After 72 hr the overlay medium was removed, monolayers washed once with phosphate buffered saline and stained with 1% crystal violet. Plaques formed by HSV at 37° were stained at 48 hr after inoculation. All plaques were counted with the aid of a stereomicroscope.

To determine plaque reduction a known number of plaque forming units (PFU) was inoculated onto cell monolayers in 35 mm dishes and overlay medium containing increasing concentrations of PAA was added. The average number of plaques counted on replicate cultures without PAA was regarded as the 100% value of plaques formed.

*Inhibition of virus by PAA-containing medium.* For multiplicity of infection (MOI) studies BB cells were grown in 16 × 125 mm tissue culture tubes and monolayers were inoculated with different multiplicities of CCV<sub>A</sub>. Maintenance medium containing increasing amounts of PAA was added, 1 ml per tube. Inoculated control tubes contained no PAA. Cultures were maintained at 25° for 1 week with daily observation for cytopathic effect (CPE). We define effective concentration of PAA as that amount of drug which completely inhibited the induction of detectable virus CPE.

*Results. Virus plaque reduction by PAA.* CCV in amounts of 200, 100 or 50 plaque forming units in separate experiments was inoculated onto confluent monolayers of BB cells in 35mm dishes. Concentrations of PAA ranging from 50 to 2000 µg/ml in overlay medium was applied for 72 hr. The resulting data listed in Table I indicates that greater than 95% of CCV<sub>A</sub> plaques were inhibited at a final drug concentration of 1000 µg/ml. This relationship remains the same whether cultures were infected with 200, 100 or 50 virus plaque forming units. Likewise, the wild-type isolate, CCV<sub>H</sub>, was similarly inhibited in the plaque reduction assay. However, plaques of this strain were reduced 100% by concentrations of 500 µg PAA/ml, half the amount required for the laboratory adapted CCV<sub>A</sub> strain.

In similar experiments using HSV, 200 PFU were inoculated onto either BHK or pRK cell cultures and concentrations of PAA in overlay medium applied for 48 hr. Table II indicates that in all cases 97% or more of both HSV-1 and HSV-2 plaques were inhibited at a final PAA concentration of 50 µg/ml.

*Effect of PAA on host cell viability.* The effect of PAA in high concentrations on BB cells was determined as follows. At the beginning of each experiment viable cell counts, as

TABLE I. CHANNEL CATFISH VIRUS PLAQUE REDUCTION BY PAA.

Virus <sup>a</sup>	PAA Conc <sup>b</sup>	No. plaques <sup>c</sup>	% Plaque reduction
CCV <sub>A</sub> 200 PFU	0	183	0
	50	175	4
	100	191	0
	200	162	11
	500	84	54
	1000	8	96
	2000	4	98
100 PFU	0	75	0
	50	71	5
	100	79	0
	200	68	9
	500	23	69
	1000	3	96
	2000	4	95
50 PFU	0	29	0
	50	24	17
	100	27	7
	200	20	31
	500	9	69
	1000	0	100
	2000	0	100
CCV <sub>H</sub> 200 PFU	0	195	0
	50	194	0
	100	134	33
	200	109	44
	500	1	100
	1000	0	100
	2000	0	100
50 PFU	0	69	0
	50	54	22
	100	28	59
	200	11	84
	500	0	100
	1000	0	100
	2000	0	100

<sup>a</sup> Channel catfish virus strains Auburn (CCV<sub>A</sub>) and Homestead (CCV<sub>H</sub>).

<sup>b</sup> In µg/ml final concentration.

<sup>c</sup> Average of four plates per PAA concentration.

calculated by trypan blue dye exclusion, were done on BB cells grown in 35 mm dishes. Representative cultures were randomly selected. Overlay medium containing PAA in final concentrations of 0, 500, and 2000 µg/ml was added to cell cultures containing no virus, and at 72 hr viable cell counts were done. The data in Table III demonstrate that the total number of viable cells was the same in PAA treated and untreated BB cell cultures. This indicates that no drug toxicity occurred during the 72 hr-CCV assay period. In addition, parallel BB cell cultures containing either 0, 500, or 2000 µg/ml of PAA were

TABLE II. HERPES SIMPLEX VIRUS PLAQUE REDUCTION BY PAA.

Cell <sup>a</sup>	PAA Conc <sup>c</sup>	No. plaques <sup>d</sup>	% Plaque reduction
BHK	0	150	0
	50	0	100
	100	0	100
	200	0	100
pRK	0	165	0
	50	0	100
	100	0	100
	200	0	100
BHK	0	199	0
	50	5	97
	100	0	100
	200	0	100
pRK	0	165	0
	50	0	100
	100	0	100
	200	0	100

Herpes simplex virus type 1 (HSV-1) strain 2bb and HSV-2) strain 196.

Primary hamster kidney (BHK) cells and primary rabbit kidney (pRK) cells.

<sup>c</sup> µg/ml final concentration.

<sup>d</sup> Average of four plates per PAA concentration.

1, trypsinized and successfully subcultured under PAA-free growth medium. *Relationship of PAA to multiplicity of infection* to determine if a PAA dose dependency for CCV similar to that reported (2) for SV, CCV<sub>A</sub> was prepared in various dilutions and inoculated onto BB cells grown in 16 culture tubes. These virus dilutions were added to multiplicities of infection of 0.1, 1.0, and 6.0 plaque forming units per cell. Maintenance media containing the PAA concentrations as listed in Table II were added to each MOI group of inoculated BB cell cultures. Viral CPE for all cultures did not progress beyond 4 days after inoculation, but cultures were observed for a period of 1 week. Results of these experiments indicated that a direct relationship does not exist between PAA concentration and viral CPE. For every tenfold increase in PAA input a twofold increase of drug was needed for total inhibition of virus cytopathology. This ranged from 500 µg PAA/ml = 0.01 PFU/cell to more than 2000 µg PAA/ml (MOI = 6.0 PFU/cell). The toxic level of PAA in BB cells was evident at 500 µg PAA/ml of culture medium.

*Host range.* The herpesviruses are widely distributed throughout animal phylogeny (17). Although they infect a range of species the

resultant interaction may vary subtly from subclinical infection to severe disease to oncogenicity. For many reasons those herpesviruses that parasitize homeothermic animals, the mammals and birds, have received most research attention. It has been consistently found that PAA in amounts of 100 µg or less inhibits the expression of each herpesvirus tested. Likewise, our results agree with the results of others (2, 4) whereby HSV-1 and HSV-2 expression at 37° is inhibited by less than 100 µg PAA/ml.

The data presented in this report support the developing contention that susceptibility to inhibition by PAA is a new characteristic of the herpesviruses. Furthermore, this characteristic is apparent in poikilothermic as well as homeothermic animal-virus systems. Our findings indicate, however, that up to 20 times the amount of drug required for other herpesvirus systems is necessary to inhibit CCV.

Currently the precise mode of virus inhibition which occurs in our system is unclear. In homeothermic systems PAA has been shown to interfere with enzymes of viral DNA replication (10, 11). In view of the vast phylogenetic distance between the mammalian and teleostean cell however, there may be differences in metabolic reactions to antiviral drugs. If the mode of action is similar then the action of PAA may be dependent upon either temperature or, relatedly, the physiology and metabolic rate of the host cell. It is well known that enzyme-substrate reactions can be directly influenced by temperature, and the importance of temperature as a catalytic mechanism has been demonstrated in the regulation of many life functions of poikilothermic species (18). The importance of host cell physiology is also suggested by the increased tolerance of BB cells to PAA. We have observed drug toxicity to occur at

TABLE III. VIABLE BB CELL COUNTS AFTER EXPOSURE TO PAA.

PAA conc <sup>a</sup>	Time <sup>b</sup>	Viable cell count <sup>c</sup>
0	0	$1.4 \times 10^6$
0	72	$1.9 \times 10^6$
500	72	$1.9 \times 10^6$
2000	72	$2.3 \times 10^6$

<sup>a</sup> In µg/ml final concentration.

<sup>b</sup> In hours.

<sup>c</sup> Trypan blue dye exclusion, total number of cells per culture.



or about the 2500  $\mu\text{g}/\text{ml}$  level as determined by loss of monolayer integrity with concurrent decrease in viable cell counts.

An alternative hypothesis is that the poikilothermic virus is itself responsible for the increased amount of drug required for inhibition of virus expression. One means to resolve this question would be a determination through a range of temperatures of PAA levels inhibiting homeothermic herpesviruses in BB cells or CCV in homeothermic cells. Unfortunately, these experiments are not now possible as BB cells will not support the replication of those broad host range homeothermic herpesviruses tested (HSV, pseudorabies virus) and CCV will only replicate in selected cells of catfish origin.

The investigation of anti-viral drugs serves a twofold purpose: The realization of potential for control of acute viral disease and the attainment of a further understanding of the mechanisms of virus host-cell interaction. A clearer insight into both these objectives may be obtained by study of the mechanism by which poikilothermic channel catfish herpesvirus is less sensitive than homeothermic herpesviruses to PAA.

**Summary.** Both the laboratory adapted Auburn strain and a recently isolated wild-type strain of channel catfish herpesvirus (CCV) were found to be inhibited by phosphonoacetic acid (PAA) when replicated in catfish cell cultures. The inhibition of virus cytopathic effect by PAA exhibited a direct relationship between the multiplicity of infection and amount of drug required. However, in this poikilothermic system up to 20 times the amount of PAA required for inhibition of homeothermic herpesvirus systems was found necessary to inhibit CCV cytopathology.

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## Effects of Indomethacin and Meclofenamate on Estrogen Induced Vasodilation in the Rabbit Uterus<sup>1</sup> (40276)

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Previous studies have shown that estrogen increases the blood flow to the pregnant and nonpregnant uterus (1-3), but the mechanism by which this vasodilation occurs has not been determined.

It has been postulated that prostaglandins mediate this vasodilation. Prostaglandins have been shown to play a role in the regulation of blood flow in the pregnant rabbit uterus (4) and some studies have indicated that prostaglandins affect blood flows in nonpregnant uteri (5). Some investigators have also reported finding increased prostaglandin synthesis in uterine tissue following estrogen treatment (6, 7).

The following experiment was designed to determine the response of the uterine vasculature to estrogen treatment in the rabbit and to determine if prostaglandins are involved in this response through the use of the known prostaglandin synthetase inhibitors, indomethacin and meclofenamate.

**Materials and methods.** Non-pregnant female New Zealand white rabbits weighing 2-3 kg were used in this study. Surgery was performed under Nembutal (Abbott Laboratories) sedation supplemented by local xylocaine (Astra Pharm.). A left ventricular catheter (0.0288 mm) was placed via the left femoral artery and a second polyvinyl catheter was inserted 8-10 cm into the left femoral vein. The femoral catheter was then led to the back incision via a subcutaneous tunnel. The catheters were secured to a packet made of medical tape and attached to the rabbit's back. Experiments were performed the following day with the awake animal resting in a restraining cage.

Mean arterial blood pressure of the rabbit was monitored with a Statham P23Db

transducer attached to the femoral catheter. Records were made with an R411 Beckman recorder with an EO-18 oscilloscope display.

Blood flows were determined by the left ventricular injection of 15 micron microspheres (3M Co., New England Nuclear) labelled with either <sup>109</sup>Gd, <sup>113</sup>Sn, <sup>85</sup>Sr or <sup>46</sup>Sc. The spheres were prepared as a suspension in 10% Dextran in saline. Each microsphere injection had a volume of 0.1-0.2 ml and contained approximately 0.5 million spheres.

**Withdrawal.** The microspheres were injected into the left ventricle while simultaneously withdrawing an integrated arterial blood sample from the femoral catheter at a rate of 2.06 ml/min for 1.5 min, starting from the time of sphere injection.

**Response to estrogen.** In five animals, the control organ blood flows were determined. A solution of 1 mg/ml beta estradiol diacetate (Sigma) in 95% ETOH was then administered at a dosage of 100 µg/kg of body weight via the left ventricle. A second determination of the organ blood flows was made 2 hr after the estrogen treatment.

**Effect of indomethacin pretreatment.** In this series seven rabbits were pretreated with a 100 mg/ml solution of indomethacin dissolved in dimethyl sulfoxide at a dosage of 20 mg/kg of body wt. Indomethacin was given 30 min prior to the control blood flow measurement, and again 30 min before the final measurement of blood flow. The effect of estrogen on the uterine blood flow was measured as described above.

**Effect of meclofenamate pretreatment.** In this series meclofenamate was administered to eight rabbits as a 20 mg/ml saline solution in a dosage of 20 mg/kg of body wt. The meclofenamate was given 30 min prior to the control blood flow measurement and again 30 min before the final measurement of blood

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flow. The effect of estrogen on the uterine blood flow was measured as described above.

**Assay.** Upon completion of the experiment, the animal was sacrificed and the uterus, kidneys and lungs were removed. Care was taken to dissect free any adipose or connective tissue from the organs. The uterus was dissected into five separate samples, and the kidneys into three samples each. Two lung samples were also taken, one sample coming from each main lobe of the lungs. Lung samples were taken for assay to determine that no shunting of microspheres across the vascular bed had occurred. The tissues were weighed and placed in counting vials. No sample vial contained tissue which extended more than 1 cm above the bottom of the vial.

Standard vials were used in assaying the samples. Each standard vial contained a known number of spheres of one of the isotopes used in the experiment embedded in wax approximately 0.5 cm from the bottom of the vial. All measurements of radioactivity were made with a three-channel, well-type, automatic  $\gamma$  counting system (Nuclear Chicago, model 1185). A standard pattern of counting the samples was used in which the standard vials were followed by the blood samples, obtained during the integrated arterial withdrawal, followed by the tissue samples. The data were printed on paper tape which was fed into a Univac 1110 computer via an interactive terminal. The data were then processed through programs developed by our laboratory. The spillovers of each isotope into the other channels was determined from the standard vials and the counts per minute per sphere were also calculated at this time. Data were reduced to counts per minute and the number of spheres in each sample. Organ blood flows and vascular resistances per gram of tissue and the ratios of test resistance to control resistance (T/C) for each tissue sample were also calculated. All results are expressed as the mean  $\pm$  the standard error of the mean. Statistical analysis included paired and un-paired *t* tests (where appropriate) to compare control and test observations.

**Dosage and vehicle.** The vehicle for the estrogen was ethanol. The dosage administered was small ( $< .35$  ml) and the measurements of blood flow were made 2 hr after the

administration of this substance. It is unlikely that the presence of ethanol was a significant factor in these experiments because ethanol was present in both the control (estrogen only) studies and in the studies using prostaglandin synthetase inhibitors. The indomethacin was administered with  $< 1$  ml of DMSO (dimethyl sulfoxide) and our observations were made after a delay of 30 min. We have examined the cardiovascular effects of DMSO in the sheep and have observed no significant cardiovascular responses to this agent 30 min after its administration.

The dose levels of indomethacin and meclofenamate were selected to ensure some degree of prostaglandin synthetase inhibition. Ryan *et al.* (5) used 20 mg/kg/day of meclofenamate and 5 mg/kg/day of indomethacin. Venuto *et al.* (8) have shown that 2 mg/kg of indomethacin or meclofenamate both reduce uterine venous prostaglandin  $E_2$  levels in pregnant rabbits.

**Results. Part 1. Responses to estrogen.** The results obtained in five rabbits are presented in Table I. Organ blood flows were measured before (Control) and 2 hr after (Test) treatment with 0.1 mg/kg estrogen. Mean arterial blood pressures were not affected by the estrogen treatment. In each of the five animals, the vascular resistance of the uterus decreased in response to estrogen. The change in mean resistance of  $192.96 \pm 32.5$  in the control state to  $36.92 \pm 8.5$  mm Hg  $\times$  min/ml  $\times$  g after estrogen, was significant ( $P < .003$ ). The renal vascular resistance was not affected by estrogen treatment.

**Part 2. Pretreatment with indomethacin.** The organ blood flows in seven rabbits which had been pretreated with 20 mg/kg indomethacin were measured both before (Control) and again 2 hr after (Test) estrogen treatment. The results are presented in Tables II and III. Mean arterial blood pressures were not affected by indomethacin pretreatment. Uterine vascular resistance was also not significantly affected by the indomethacin. The renal vascular resistance increased from a mean control value of  $24.63 \pm 3.0$  to  $38.51 \pm 5.2$  mm Hg  $\times$  min/ml  $\times$  g (Table III). This was a significant increase ( $P < .04$ ) due to indomethacin pretreatment.

Following indomethacin pretreatment, mean arterial blood pressures were not af-

fects by estrogen treatment. Uterine vascular resistance decreased from a control value of  $299.13 \pm 69.1$  to  $137.90 \pm 47.3$  mm Hg  $\times$  min/ml  $\times$  g ( $P < .004$ ) after pretreatment with estrogen (Table II). The renal vasculature was not affected by the estrogen treatment.

Comparisons were made of the resistance ratios (T/C) between normal rabbits and rabbits which had been pretreated with indomethacin to determine any effect which indomethacin might have on the vascular response to estrogen treatment (Table IV). The untreated uterus had a mean T/C value of

TABLE I.<sup>a</sup>

Animal	Resistance (mm Hg $\times$ min)/ml $\times$ g							
	Blood pressure (mm Hg)		Uterine resistance			Renal resistance		
	C	T	C	T	T/C	C	T	T/C
1	90	92	216.48	50.29	0.233	13.37	25.48	1.905
2	112	102	231.56	53.29	0.231	29.26	24.66	0.843
3	88	86	104.22	11.00	0.106	23.09	15.11	0.654
4	80	86	279.98	47.42	0.169	28.32	27.53	0.972
5	80	92	132.57	22.48	0.170	29.09	30.77	1.058
Mean	90	92	192.96	36.92	0.182	24.62	24.71	1.086
SEM	$\pm 7$	$\pm 3$	$\pm 32.5$	$\pm 8.5$	$\pm 0.02$	$\pm 3.0$	$\pm 2.6$	$\pm 0.24$
	NS		$P < .003$			NS		

<sup>a</sup> The uterine and renal vascular resistance per gram of tissue of five rabbits before (C) and 2 h after (T) the administration of 0.1 mg/kg estrogen. Mean arterial blood pressures and resistance ratios (T/C) are also given.

TABLE II.<sup>a</sup>

Animal	Resistance (mm Hg $\times$ min)/ml $\times$ g							
	Blood pressure (mm Hg)		Uterine resistance			Renal resistance		
	C	T	C	T	T/C	C	T	T/C
1	76	108	220.88	218.16	0.988	23.65	77.01	3.256
2	88	80	233.67	33.79	0.145	33.50	22.59	0.674
3	90	88	143.72	37.01	0.257	30.45	30.35	0.997
4	100	94	543.48	302.89	0.557	41.38	44.44	1.074
5	98	106	575.03	283.85	0.494	65.52	85.73	1.308
6	80	76	136.67	59.81	0.437	31.89	21.71	0.681
7	108	100	240.43	29.77	0.124	43.17	38.56	0.893
Mean	91	93	299.13	137.90	0.429	38.51	45.77	1.269
SEM	$\pm 4$	$\pm 5$	$\pm 69.1$	$\pm 47.3$	$\pm 0.11$	$\pm 5.2$	$\pm 9.9$	$\pm 0.34$
	NS		$P < .004$			NS		

<sup>a</sup> The uterine and renal vascular resistances per gram of tissue of seven rabbits pretreated with 20  $\mu$ g/kg indomethacin before (C) and 2 h after (T) the administration of 0.1 mg/kg estrogen. Mean arterial blood pressures and resistance ratios (T/C) are also given.

TABLE III.<sup>a</sup>

	Resistance (mm Hg $\times$ min)/ml $\times$ g					
	Blood pressure (mm Hg)		Uterine resistance		Renal resistance	
	N	P	N	P	N	P
Mean	90	91	192.96	299.13	24.63	38.51
SEM	$\pm 7$	$\pm 4$	$\pm 32.5$	$\pm 69.1$	$\pm 3.0$	$\pm 5.2$
N	5	7	5	7	5	7
	NS		NS		$P < .04$	

<sup>a</sup> A comparison of the uterine and renal vascular resistance per gram of tissue during the control period of five normal (N) rabbits and seven rabbits pretreated (P) with 20 mg/kg indomethacin. A comparison of the mean arterial blood pressures is also provided.

0.182 which differed significantly ( $P < .05$ ) from the pretreated T/C value of 0.429. Indomethacin depressed the uterine response to estrogen.

*Part 3. Pretreatment with meclofenamate.* The organ blood flows in eight animals pre-

treated with 20 mg/kg meclofenamate were measured both before (Control) and again 2 hr after (Test) estrogen treatment. The results are presented in Tables V and VI. Meclofenamate pretreatment had no effect on the mean arterial blood pressure. The uterine vascular resistance increased from a mean control value of  $192.96 \pm 32.5$  to  $416.42 \pm 72.6$  mm Hg  $\times$  min/ml  $\times$  g ( $P < .02$ ) following the meclofenamate treatment. The renal vascular resistance significantly increased from  $24.63 \pm 3.0$  to  $40.33 \pm 6.1$  mm Hg  $\times$  min/ml  $\times$  g ( $P < .04$ ) after pretreatment with meclofenamate (Table VI).

Following pretreatment with meclofenamate the mean arterial blood pressure was not affected by estrogen treatment. The uterine vascular resistance significantly decreased from a mean value of  $416.42 \pm 72.6$  before estrogen to  $69.58 \pm 21.8$  mm Hg  $\times$  min/ml  $\times$  g ( $P < .001$ ) after estrogen (Table V). The renal vasculature was not affected by the

TABLE IV.<sup>a</sup>

	Resistance ratios			
	Normal T/C	Indometh- acin pre- treatment T/C	Normal T/C	Meclofen- amate pre- treatment T/C
Mean	0.182	0.429	0.182	0.158
SEM	$\pm 0.02$	$\pm 0.11$	$\pm 0.02$	$\pm 0.03$
N	5	7	5	8
	$P < .05$		NS	

<sup>a</sup> The effect of 0.1 mg/kg estrogen on the uterine vasculature of five normal rabbits and rabbits pretreated with either 20 mg/kg indomethacin or 20 mg/kg meclofenamate. The data are expressed as ratios (T/C) of the resistance 2 hr after estrogen treatment (T) to that seen before administration of the estrogen (C).

TABLE V.<sup>a</sup>

Animal	Blood pressure (mm Hg)		Resistance (mm Hg $\times$ min)/ml $\times$ g					
			Uterine resistance			Renal resistance		
	C	T	C	T	T/C	C	T	T/C
1	84	82	685.06	28.30	0.041	38.99	33.75	0.866
2	80	92	298.94	60.84	0.204	25.94	26.20	1.010
3	86	86	315.82	47.82	0.151	37.40	36.78	0.984
4	86	80	674.91	198.78	0.295	73.20	94.04	1.280
5	108	100	611.76	122.55	0.200	57.13	61.48	1.070
6	82	93	172.58	16.89	0.098	25.01	26.66	1.066
7	88	90	268.15	25.74	0.096	41.13	51.47	1.251
8	90	102	304.17	55.69	0.183	23.81	29.71	1.248
Mean	88	91	416.42	69.58	0.158	40.33	45.01	1.097
SEM	$\pm 3$	$\pm 3$	$\pm 72.6$	$\pm 21.8$	$\pm 0.03$	$\pm 6.1$	$\pm 8.3$	$\pm 0.05$
	NS		$P < .0005$			NS		

<sup>a</sup> The uterine and renal vascular resistances per gram of tissue of eight rabbits pretreated with 20  $\mu$ g/kg meclofenamate before (C) and 2 hr after (T) the administration of 0.1 mg/kg estrogen. Mean arterial blood pressures and resistance ratios (T/C) are also given.

TABLE VI.<sup>a</sup>

	Blood pressure (mm Hg)		Resistance (mm Hg $\times$ min)/ml $\times$ g			
			Uterine resistance		Renal Resistance	
	N	P	N	P	N	P
Mean	90	88	192.96	416.42	24.63	40.33
SEM	$\pm 7$	$\pm 3$	$\pm 32.5$	$\pm 72.6$	$\pm 3.0$	$\pm 6.1$
N	5	8	5	8	5	8
	NS		$P < .02$		$P < .04$	

<sup>a</sup> A comparison of the uterine and renal vascular resistance per gram of tissue during the control period of five normal (N) rabbits and eight rabbits pretreated (P) with 20 mg/kg meclofenamate. A comparison of the mean arterial blood pressures is also provided.

on treatment. Meclofenamate did not alter the uterine vascular response to estrogen.

**Discussion.** It has been postulated that estrogen-induced uterine vasodilation is mediated via a biochemical chain of events initiated by estrogen receptor binding and consequent to the synthesis of new mRNA and, subsequently, the synthesis of new protein (1). Killam *et al.* (10) have described the effect of estrogen on the sheep uterus and indicated a possible release of acetylcholine or histamine as the intermediate step in the chain of events. Clark *et al.*, however, determined that the administration of uterine receptor antagonists has no effect on estrogen induced increases in uterine volume (11). Resnik *et al.* (12) also concluded that acetylcholine, isoproterenol and histamine are not mediators of the response to estrogen and proposed the release of a small polypeptide such as bradykinin or an enzyme which has a role in the biosynthesis of adenosine and its release (2). Several studies have indicated an increase in the synthesis of uterine prostaglandins after estrogen treatment (5-7). Ryan *et al.* have shown that prostaglandins exhibit properties concurrent with the hypothesis that prostaglandins mediate estrogen induced hyperemia (5). They showed that blocking prostaglandin synthesis with both indomethacin and meclofenamate depressed the uterine response to estrogen in rats. Castracane and Jordan, however, have found that inhibiting protein synthesis and thereby blocking the biological chain of events leading to the hyperemic response, had no effect on the estrogen induced increase in prostaglandins (13). They concluded that the production of prostaglandins by the uterus in response to estrogen may be a function of estrogen mediated to its function as an initiator of estrogen induced hyperemia.

This study was designed to determine whether prostaglandin synthesis is a necessary step in the mediation of the estrogenic response. The experimental data presented in this paper indicate that estrogen induced vasodilation is not mediated by prostaglandin synthesis. The vasoconstriction shown to be occurring in the kidneys following treatment with either indomethacin or meclofenamate suggests that prostaglandin synthesis blockage occurred in concordance with the study

by Malik and McGiff on prostaglandin modulation of vascular resistance in rabbit kidneys (14). The uterus showed no vasoconstriction due to indomethacin so that the vasoconstriction seen after meclofenamate may have been due to a side effect of the drug. The fact that indomethacin depressed the uterine response to estrogen is in concordance with the literature, but must be examined in view of the fact that meclofenamate did not produce a similar response. It is our conclusion that the indomethacin induced depression of the response to estrogen was not due to the blockade of prostaglandin synthesis, but due to a side effect of indomethacin or its vehicle. Therefore, prostaglandin synthesis does not appear to be essential to estrogen induced vasodilation in the rabbit uterus.

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# Superoxide Dismutase in Bovine Fetal Ductus Arteriosus, Thoracic Aorta, and Pulmonary and Umbilical Arteries (40277)

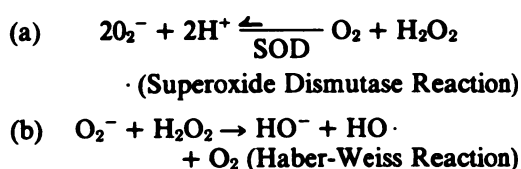
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Soon after birth the lumens of the ductus arteriosus and the umbilical artery are obliterated. Some researchers have suggested that oxygen toxicity, resulting from the increased arterial oxygen tension occurring after birth and the development of an "active hypersensitivity" reaction to oxygen prior to birth, is the cause of widespread intracellular and extracellular destruction noted in the subintimal regions of the media (muscular layer), as well as the intimal layer itself in the ductus arteriosus (1, 2). This is similar to the explanation offered for the closure of premature infants' retinal arteries and consequent retrolental fibroplasia and blindness from exposure to excessively high oxygen levels in the hyperbaric chamber (1, 3). Other workers (4-6) have presented histological and other evidence that the ductus arteriosus of guinea pig, rabbit, rat, and mouse fetuses allowed to breathe showed widespread intracellular and matrix destruction in the histological regions previously mentioned; such changes were not noted in fetuses frozen without permitting respiration.

A suitable explanation for the temporal relationship between increasing arterial oxygen tensions and cellular degeneration could certainly be superoxide radicals, hydroperoxides, and hydroxyl radicals, all powerful oxidants which are destructive in biological systems of cells existing in aerobic conditions (3, 7, 8). It has been demonstrated that rats exposed to gradually increasing levels of  $O_2$  in their environment had significantly greater levels of SOD in lung tissue and survived for significantly longer periods of time after exposure to toxic levels of  $O_2$  than did control rats (7, 8). We postulated that an overproduction of hydroxyl radicals could result after an increase in oxygen tension in tissues such as the ductus arteriosus and umbilical artery, if such tissues possessed lower levels of SOD, as compared with such permanent tissues as

the pulmonary artery and thoracic aorta. Superoxide dismutase catalyzes the dismutation of two molecules of the superoxide anion forming one molecule each of oxygen and hydrogen peroxide, while the superoxide anion and hydrogen peroxide can react to form the hydroxyl radical:



SOD functions to remove one of the reactants of the Haber-Weiss reaction, and this enzyme has been extensively studied by McCord and Fridovich (3), as well as by others.

In this study we chose to examine the levels of SOD in several tissues of bovine fetuses, using an enzymic activity assay and a radial immunodiffusion assay. There were two groups of two tissues used for comparative purposes; the two fetal blood vessels which obliterate after birth, the ductus arteriosus and umbilical artery, and the two blood vessels which do not obliterate after birth, the pulmonary artery and the thoracic aorta. Reported herein are the results of this study which we feel are in support of our hypothesis.

**Materials and methods.** A local meat packing firm allowed us access to fetal calves approximately forty minutes following the killing of the mother. Gestational ages of the calves were estimated using such criteria as crown-rump length, body hair patterns, and the presence of erupted incisor teeth (9). Eighty percent of the calves in this study were "full-term" by the criteria mentioned. The thoracic cavity was then entered and an *en bloc* excision of the heart, great vessels, and the entire length of the thoracic aorta was performed; additionally, a small segment of

ical artery was obtained from the umbilical cord. The great vessels were then identified, dissected free, excised, washed three times in 0.15 M NaCl, immediately frozen on dry ice and stored for 2 weeks at  $-30^{\circ}$ . The tissues were then thawed, washed again three times in 0.15 M NaCl, homogenized in 4 vol 0.15 M NaCl with a Tenbroeck glass homogenizer, and centrifuged at 100,000g for 1 h at  $2^{\circ}$ . The supernatant was then used for analysis of SOD activity. If there is significant blood in the prepared tissue, this will contribute to the total SOD activity of the tissue as erythrocytes do possess significant levels of SOD. Whole blood was obtained from four fetal calves in the study. The erythrocytes were lysed with an equal volume of distilled water and the solution then reprecipitated into 0.15 M NaCl. The lysed erythrocyte supernatant was then centrifuged and the supernatant was diluted with 0.15 M NaCl to 1:10 solutions with hemoglobin concentrations in ranges comparable to the tissue supernatants from the blood vessel preparations. Hemoglobin levels of the lysed erythrocytes and blood vessel supernatants were measured at  $24.7 \text{ cm}^{-1}$  using a Cary spectrophotometer. SOD activity of the erythrocyte supernatants was measured. SOD activity was measured using the xanthine oxidase-cytochrome c assay of McCord and Fridovich (10). Bovine erythrocyte SOD was purified to electrophoretic homogeneity by the method of McCord and Fridovich (10). This preparation was used to prepare standards in rabbits. Immune rabbit  $\gamma$ -globulins were isolated as previously described, and these were used to determine the levels of SOD using a radial immunodiffusion assay. Bovine xanthine oxidase was purified to electrophoretic homogeneity from raw cream (12). Protein concentrations were determined by the method of Lowry *et al.* (13).

Acidic HCl and base and cytochrome c were obtained from the Sigma Chemical Company. Agar was obtained from Difco Company. All other chemicals were reagent grade.

**Results and discussion.** The results of enzymic and immunochemical assays for SOD in tissues from thirteen bovine fetuses are presented in Table I. In all individual fetuses the levels of SOD determined in the

four tissues indicated that the ductus arteriosus and umbilical artery were always lower than the pulmonary artery and thoracic aorta although a comparison between animals did not always follow this pattern.

The data were compared using the "t" test of significance and the results of such comparisons are shown in Table II. As can be seen, in nearly all comparisons the levels of SOD in the ductus arteriosus and umbilical artery were statistically significantly lower than those found in the pulmonary artery and thoracic aorta. The level of SOD in the ductus arteriosus and the umbilical artery were not statistically significantly different from each other. Likewise, the levels of SOD in the pulmonary artery and thoracic aorta were not statistically significantly different from each other.

Erythrocytes do contribute to the SOD activity of tissue extracts although this contribution is negligible if it is possible to wash the tissues relatively free from blood (7). In this study the hemoglobin in the tissue supernatants was in the range of  $1-2 \times 10^{-6} \text{ M}$ .

TABLE I. BOVINE FETAL SUPEROXIDE DISMUTASE.

Tissue	Activity enzyme units <sup>a</sup>	Radial immunodiffusion $\mu\text{g}^b$
	mg protein	mg protein
Ductus Arteriosus	$2.32 \pm 0.33^c$	$77.8 \pm 5.8^c$
Umbilical Artery	$1.97 \pm 0.16$	$80.2 \pm 7.8$
Pulmonary Artery	$3.64 \pm 0.32$	$94.7 \pm 6.7$
Thoracic Aorta	$3.45 \pm 0.31$	$113.9 \pm 8.3$

<sup>a</sup> Determined with xanthine oxidase-cytochrome c assay, expressed per mg cytosolic protein.

<sup>b</sup> Expressed as  $\mu\text{g}$  superoxide dismutase per mg cytosolic protein.

<sup>c</sup> Values are expressed as the mean  $\pm$  SE for thirteen samples run in duplicate.

TABLE II. "t" TEST OF SIGNIFICANCE.<sup>a</sup>

Paired tissues	Enzyme assay	RID
DA-PA	0.001	0.01
DA-TA	0.05	0.01
UA-PA	0.001	0.05
UA-TA	0.001	0.02
DA-UA	0.4	0.7
PA-TA	0.7	0.2

<sup>a</sup> The data of Table I were analyzed by pairing the indicated tissues. The confidence levels are indicated for the two types of assay, enzymic and immunochemical (RID, radial immunodiffusion).



The SOD activity of the lysed erythrocyte supernatants in this hemoglobin concentration range was negligible (less than one percent). Additional evidence to discount the contribution of erythrocytes in this study is noted in that the hemoglobin concentrations varied randomly in the tissue samples and did not correlate with the differences between the SOD activity of the blood vessel preparations.

Undoubtedly the etiology of ductus arteriosus closure is multifactorial, and it is not possible here to elaborate the numerous mechanisms proposed (14-16). It is helpful to view ductus arteriosus closure as both a physiological and anatomical event; that is to say, the ductus arteriosus responds to varying oxygen tensions and hemodynamic changes by changing its lumen size *in situ*, and it undergoes obliterative fibrotic changes to ultimately become the ligamentum arteriosum in the usual case. The *in vitro* responsiveness of this vessel to varying oxygen tensions has been consistently reported in the literature. The role of prostaglandins in the closure of the ductus arteriosus is also of current interest (17-19).

This study suggests that a deficiency of SOD could contribute to the degenerative cellular changes presumed to occur as part of the obliterative process in the bovine ductus arteriosus and umbilical arteries. Further studies need to be conducted to determine if the rise in arterial oxygen tension at parturition is sufficient to create the oxidant stress this study is proposing. In addition we are not capable of ascertaining the distribution of SOD across the wall of the tissues we have examined, which might be of importance in the degeneration of the ductus arteriosus and umbilical artery. Perhaps in the future a histological stain for SOD of sufficient sensitivity will be developed and can be used to answer such questions.

The levels of SOD seen in the ductus arteriosus and in the umbilical artery are 54-67% (activity assay) and 68-84% (RID assay) of the levels found in the pulmonary artery and in the thoracic aorta. Michelson *et al.* (20) have suggested that "levels of less than 50% of the normal mean for superoxide dismutase are more or less lethal due to the increased toxicity of uncontrolled superoxide." This contention was based on a survey

of SOD activities in a cross section of the human population in France, comparing normal and abnormal populations. Extremely low levels of SOD correlated in several cases with associated physical and mental problems. The ability of a newborn to handle an increased flux of superoxide, consequent to exposure to increased oxygen tensions, may reflect the absolute and quantitative amounts of SOD present in particular tissues. Those with high levels of SOD will survive, and those with low levels of SOD will degenerate.

**Summary.** Soon after birth the lumens of the ductus arteriosus (DA) and umbilical artery (UA) are obliterated. It has been suggested that oxygen toxicity, resulting from an increased oxygen tension, is the cause of this destruction with superoxide radicals and hydroxyl radicals being implicated as mediators. A deficiency of superoxide dismutase (SOD) in these tissues was hypothesized as being responsible for an increase in the levels of superoxide and hydroxyl radicals. SOD levels were determined enzymatically and immunochemically in four tissues obtained from thirteen bovine fetuses. SOD levels in the DA and UA were found by both assays to be statistically significantly lower than that found in such permanent vessels as the pulmonary artery and thoracic aorta. These data are in support of the hypothesis that a lower level of SOD in the ductus arteriosus and umbilical artery may contribute to the rapid deterioration of these tissues upon exposure to greatly increased oxygen tensions.

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# DUCTUS ARTERIOSUS SUPEROXIDE DISMUTASE

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## Mouse Hepatitis Virus (MHV) Infection in Thymectomized C<sub>3</sub>H Mice (40278)

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The macrophage plays a crucial role in the genetic susceptibility of mice to develop fatal hepatitis when infected with mouse hepatitis virus (MHV) (1). The adult C<sub>3</sub>H mice do not die after inoculation of MHV grown in Princeton mice (MHV(PRI)) and their macrophages do not support MHV(PRI) multiplication. On the other hand, infant C<sub>3</sub>H and infant and adult Princeton (PRI) mice develop a fatal infection after inoculation with MHV(PRI) and their macrophages support the multiplication of this virus and are destroyed by it (2). Intermediate susceptibility of macrophages *in vitro* is associated with virus persistence *in vivo* (3). A variety of treatments which depress the cell mediated immune response makes the MHV infection pathogenic for the genetically resistant mouse. Adult C<sub>3</sub>H mice develop fatal MHV infection after neonatal thymectomy (4) or after treatment with cortisone (5), cytoxan (6) or preinfection with *Eperythrozoon coccoides* (7) and adult A strain mice are rendered susceptible to fatal MHV infection by a variety of treatments such as x-irradiation, administration of antilymphocyte serum and neonatal thymectomy (8, 9).

We report here that although the outcome of MHV(PRI) inoculation in adult PRI and C<sub>3</sub>H mice is very different, both strains are infected by the same minimal infectious dose of MHV(PRI). In addition, although MHV(PRI) infection is fatal both for adult neonatally thymectomized C<sub>3</sub>H mice and the genetically susceptible PRI mice, the course of infection in these two strains is quite dissimilar. These findings suggest that the routine recovery of adult C<sub>3</sub>H mice from MHV(PRI) infection requires both virus resistant macrophages and normal thymic function.

**Materials and methods.** *Virus.* MHV-2 strain of virus originally obtained from Dr. John Nelson (10) was maintained in our laboratory by intraperitoneal (ip) inoculation of

4 week old PRI mice. This strain is referred to as MHV(PRI). A variant, MHV(C<sub>3</sub>H) which was derived from MHV(PRI) but which is lethal for both C<sub>3</sub>H and adult PRI mice (11) was maintained by ip inoculation of 4-week old C<sub>3</sub>H mice. The stock virus preparations were 10% homogenates of livers from virus infected mice. Titrations were performed by inoculation of 0.2 ml of serial tenfold dilutions in each of three tubes of cultured peritoneal macrophages from PRI mice prepared as described previously (12), but maintained in Eagle's minimum essential medium (Earle's salts) supplemented with 20% calf serum (FCS). The cultures were observed for viral cytopathic effect (CPE) for 8 days. The 50% tissue culture infectious dose (TCID<sub>50</sub>) was calculated by the method of Reed and Muench (13).

**Mice.** Three strains of inbred mice, C<sub>3</sub>H and C<sub>3</sub>Hss were used (12, 14, 15). The C<sub>3</sub>Hss strain is congenic with the C<sub>3</sub>H strain but is susceptible to fatal infection with MHV(PRI) and its macrophages support MHV (PRI) multiplication. It was developed by introducing the PRI gene for susceptibility to MHV(PRI) into C<sub>3</sub>H mice (15). Mice were infected by the ip route. Thymectomy was performed on C<sub>3</sub>H mice within 24 hr of birth. Both left and right sections of the thymus were removed by gentle suction. Thymectomized mice were infected at 4-6 weeks of age. Sham operated mice served as controls.

**Immunofluorescence.** Anti-serum was prepared in vaccinated PRI mice. PRI mice were inoculated for four successive weeks with propiolactone inactivated vaccine (6), challenged with live virus and bled one week later. The serum was stored at -20°. Sections were cut on a cryostat at 4 microns. Fluorescein conjugated goat anti-mouse serum (concentration 1:10 (Meloy Laboratories, Springfield, VA) was used with a counterstain of Evans Blue prepared as a 0.5% stock

and used at a 1:8 concentration. *Pathology.* Sections of liver were placed in 10% buffered formalin, cut and stained with hematoxylin and eosin for histopathol-

*Results. Infectivity and pathogenicity of MHV(PRI) for PRI and C<sub>3</sub>H mice.* MHV(PRI) was titrated in 4-6 week old PRI and C<sub>3</sub>H mice using four mice per dilution and inoculated mice were observed for 7 days postinfection. On day 7, the surviving mice of the titrations were challenged ip with 2.6 units of MHV(C<sub>3</sub>H) virus. Ability of mice to resist MHV(C<sub>3</sub>H) challenge was as evidence that it was previously infected with MHV(PRI).

MHV(PRI) had a LD<sub>50</sub> titer of 10<sup>8.3</sup> in PRI mice (Table I). None of the PRI mice of the titration resisted MHV(C<sub>3</sub>H) challenge indicating that MHV(PRI) did not induce a nonfatal immunizing infection in PRI mice. In contrast MHV(PRI) produced 100% mortality in C<sub>3</sub>H mice inoculated with 10<sup>-8.0</sup> dilutions of the virus and all but one of the survivors of this titration resisted challenge with MHV(C<sub>3</sub>H). The immunizing effect of the virus was 10<sup>8.0</sup> for C<sub>3</sub>H mice. These results indicate that PRI and C<sub>3</sub>H mice were equally susceptible to infection with MHV(PRI) but that the infection was uniformly fatal in PRI and uniformly nonfatal in C<sub>3</sub>H mice.

*Course of MHV(PRI) infection in thymectomized C<sub>3</sub>H mice.* Neonatally thymectomized or sham operated C<sub>3</sub>H mice were inoculated ip with 10<sup>5.0</sup> TCID<sub>50</sub> of MHV(PRI) and observed for mortality, virus titers in blood and liver pathology. All thymectomized mice were checked for the completeness of thymectomy at the time they died or were sacrificed and animals with thymus remnants excluded from the study.

Of the 13 mice infected with MHV(PRI) and also completely thymectomized, the mortality was 100% (Fig. 1). This was in contrast

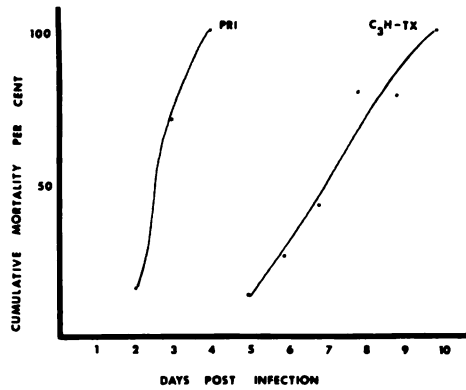


FIG. 1. Cumulative mortality of PRI and C<sub>3</sub>H thymectomized mice infected with MHV(PRI) virus. Data on PRI mortality were pooled from several experiments and include over 100 mice which were inoculated with similar virus dilutions. Data on C<sub>3</sub>H-thymectomized mice are based on 13 completely thymectomized mice.

to the 0% mortality in intact C<sub>3</sub>H mice which were inoculated with MHV(PRI). Deaths occurred between day 5 and 10 after inoculation of virus with an average survival time of between 7 and 8 days. This timing of mortality was very different from that in PRI mice which, while they also have a mortality of 100%, survive only 2-3 days postinfection (Fig. 1).

Virus titers in livers of thymectomized and sham operated C<sub>3</sub>H mice are shown in Fig. 2. Until day 7 postinfection, the titers in the two groups were comparable and ranged between 10<sup>5.0</sup> and 10<sup>7.5</sup>. Exceptions to this were two sham operated mice which had titers between 10<sup>2.0</sup> and 10<sup>3.0</sup> on day 6. After day 7, there was a marked reduction in liver titers of sham operated mice. Of eight livers titrated between days 8 and 12, seven were negative for virus and the eighth had a titer of <10<sup>1.0</sup>. In contrast, virus titers in completely thymectomized mice continued to remain high; all of four livers harvested from this group between days 8 and 10 had titers between 10<sup>5.0</sup> and 10<sup>7.0</sup>.

The pathologic lesions in sham operated and thymectomized C<sub>3</sub>H mice were very similar until day 5. By day 4 the livers showed general architectural disruption with coagulative change with diffuse and focal inflammation in which polymorphonuclear leukocytes were most prominent. Eosinophilic bodies as described by Ruebner and Miyai (16) could be seen in areas of necrosis. The liver

TABLE I. INFECTIVITY AND PATHOGENICITY OF MHV(PRI) FOR 4-6 WEEK OLD PRI AND C<sub>3</sub>H MICE.

Mouse	MHV(PRI) virus	
	Infectious dose <sub>50</sub>	Lethal dose <sub>50</sub>
PRI	10 <sup>8.0</sup>	<10 <sup>2.0</sup>
thymectomized	10 <sup>8.2</sup>	10 <sup>8.2</sup>

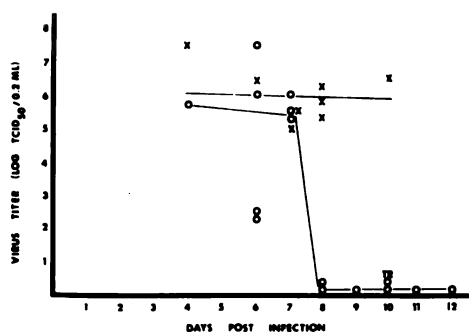


FIG. 2. Virus titers in livers from virus infected sham operated C<sub>3</sub>H and thymectomized C<sub>3</sub>H mice on PRI macrophage cultures. This graph includes the results of several experiments. ○ = liver from one C<sub>3</sub>H sham operated mouse; × = liver from one C<sub>3</sub>H thymectomized mouse, TR = Trace of virus, only one of three cultures inoculated with lowest dilution affected.

sections from sham operated C<sub>3</sub>H mice taken on day 6 showed perivascular infiltration of mainly mononuclear cells with small foci of mononuclear cells on top of necrotic parenchymal cells. The livers of C<sub>3</sub>H thymectomized mice of the same time period had large areas of acute fulminating lesions with tissue hemorrhage, necrotic debris, and the presence of polymorphonuclear leukocytes and mononuclear cells. On day 8, liver sections of sham operated C<sub>3</sub>H mice showed a few focal areas of resolving lesions, while liver sections from C<sub>3</sub>H thymectomized mice showed foci of degeneration with larger foci showing centers of liquefaction. Sections of livers of sham operated C<sub>3</sub>H mice from 9 through 14 days were normal with the exception of two mice whose livers showed occasional resolving lesions. In summary, liver sections from sham operated C<sub>3</sub>H controls showed focal hepatitis with subsequent recovery, whereas liver sections from thymectomized C<sub>3</sub>H mice showed focal hepatitis progressing to diffuse hepatitis with no recovery. The sham operated mice described above had more severe pathologic lesions and higher virus titers in livers than what is ordinarily found in normal C<sub>3</sub>H mice infected with MHV(PRI). The reason for this was not clear.

We also compared the pattern of viral multiplication in livers of PRI, C<sub>3</sub>H and C<sub>3</sub>Hss mice by histopathology, immunofluorescence and viral titrations. Mice were infected ip with  $10^{5.0}$  TCID<sub>50</sub> of MHV(PRI) and were

sacrificed at 3, 6, 10, 24, 48 and 72 hr infection. Liver sections from PRI showed increased cellular infiltration hr which progressed to necrosis of parenchymal cells with eosinophilic bodies by and extensive tissue destruction with hemorrhage by 72 hr. Immunofluorescence was detected and observed to spread as the liver and cellular destruction grew. In C<sub>3</sub>H an infiltration of mononuclear cells was detected as early as 6 hr postinfection. necrotic foci were observed by 48 hr. In liver sections very little fluorescence was noted in the first 10 hr. Small foci of necrosis containing eosinophilic bodies and Kupffer cells were apparent by 48 hr. There was a striking difference in growth between the susceptible (PRI, C<sub>3</sub>H) and resistant (C<sub>3</sub>Hss) mice (Fig. 3). Virus in PRI livers were higher than those in C<sub>3</sub>H livers by 2 log<sub>10</sub> units by 24 hr and this difference increased to 6 log<sub>10</sub> units by 72 hr. The C<sub>3</sub>Hss mice resembled PRI mice with respect to both virus titers in liver and pattern of mortality.

*Growth of MHV(PRI) in macrophage cultures from thymectomized and nonthymectomized C<sub>3</sub>H mice.* Earlier work has shown the pathogenic effect of MHV(PRI) on mouse was closely correlated with the number of the peritoneal macrophage of that mouse to support multiplication of the virus (1). It was therefore of interest to see if cultures of macrophages derived from thymectomized C<sub>3</sub>H mice supported growth of MHV(PRI).

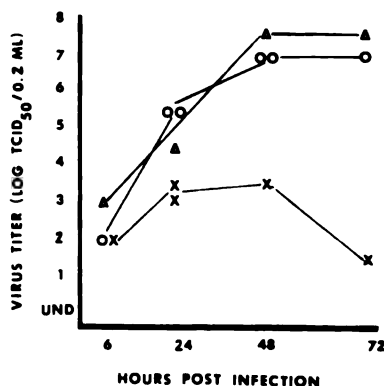


FIG. 3. Virus titers in livers of PRI, C<sub>3</sub>H and C<sub>3</sub>Hss mice in the first 3 days after infection with MHV(PRI). ○ = PRI mice. × = C<sub>3</sub>H mice. △ = C<sub>3</sub>Hss mice.

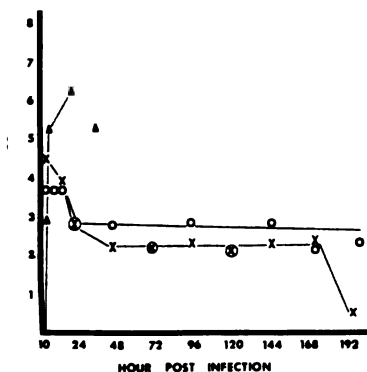
phages were removed from 4 to 6 week thymectomized and nonthymectomized mice. Only those thymectomized mice which had no grossly visible thymus were used as macrophage donors. Infected cells containing  $10^{8.0}$  infective units of virus were allowed to adsorb for 30 min and the cells were then washed and fresh medium added. The tubes of cultures were observed daily for CPE and harvested at various times after infection. They were stored frozen at  $-70^{\circ}$  until they were titrated on macrophages from PRI mice.

The virus did not produce CPE in either intact C<sub>3</sub>H thymectomized macrophage cultures and there was no difference between intact macrophages in their ability to support MHV(PRI) multiplication (Fig. 4). Low titers of virus were recovered from both kinds of macrophages through the observation period of 192 hrs. This pattern of virus growth in C<sub>3</sub>H macrophages was markedly different from that in PRI macrophages (Fig. 4), in which the virus grows rapidly with complete lysis of cells in 48 hr.

**Discussion.** Our studies confirm the pre-reports of Stutman and Yunis (4) and Provost and his colleagues (8, 9) that thymectomy increases the pathogenicity of MHV(PRI) in genetically resistant mice. MHV(PRI) produced no mortality in the intact C<sub>3</sub>H mouse but it infected this strain as readily as it did the PRI mouse. Thymectomy increased the mortality in the C<sub>3</sub>H mouse

from 0 to 100%. The pathologic studies as well as virus titers in the liver clearly indicated that the death of the thymectomized C<sub>3</sub>H mouse was due to its inability to resolve the early hepatic lesions which occurred in both thymectomized and nonthymectomized animals. These lesions progressed to fulminant hepatitis in the thymectomized C<sub>3</sub>H mouse resulting in death about 6–10 days after inoculation of virus, but were completely resolved in the intact C<sub>3</sub>H mouse. This requirement of thymic function for the recovery of C<sub>3</sub>H mice from MHV hepatitis appears to be similar to that described by Blanden (17) for the resolution of ectromelia infection of mice.

Although the MHV(PRI) infection was uniformly fatal in thymectomized C<sub>3</sub>H mice as well as in PRI mice, the course of the disease was very different in these two strains. In PRI mice the virus multiplies very rapidly leading to death in 2–3 days whereas in the thymectomized C<sub>3</sub>H mice mortality occurred later and over a longer period. This difference very likely reflects the fact that the PRI macrophages support very well the multiplication of MHV(PRI) while the macrophages of thymectomized C<sub>3</sub>H mice do not. The C<sub>3</sub>Hss mouse resembled the PRI mouse in its susceptibility to MHV(PRI). Differences in survival time after MHV infection has been shown even among susceptible strains of mice (18). This difference in susceptibility was related to the varying ability of the macrophages as the primary targets of the virus to support viral growth. These observations indicate that resistance to MHV(PRI) infection, as in the intact adult C<sub>3</sub>H mouse, requires *both* a resistant macrophage which limits the spread and multiplication of the virus and an intact thymic function which is necessary for the resolution of focal hepatic lesions. In mice that have susceptible macrophages, namely, infant C<sub>3</sub>H, infant and adult PRI, and the congenic C<sub>3</sub>Hss strain (15), the infection is so overwhelming that it kills the mouse before it has had a chance to develop an effective T cell response. In experiments not described here, transfer of immune C<sub>3</sub>H spleen cells to C<sub>3</sub>Hss animals with susceptible macrophages failed to confer resistance to challenge with MHV(PRI) (19). MHV(PRI) infection of C<sub>3</sub>H mice can also be made more pathogenic



4. Growth of MHV(PRI) in PRI, C<sub>3</sub>H and thymectomized C<sub>3</sub>H macrophage cultures. Data for PRI macrophages taken from Shif and Bang 1970 (11). Δ = PRI macrophages. ○ = C<sub>3</sub>H macrophages. × = thymectomized C<sub>3</sub>H macrophages.

by treatment of these mice with cortisone (5) or cytoxan (6). The mechanism by which these drugs bring about this effect is not clear but it could be by their destruction of T cells *per se*, or as suggested by Weiser and Bang (20), by release of lymphokines which alter macrophage susceptibility. LeBlond et al (21) have shown that both macrophages and T cells are necessary in the transfer of resistance to MHV to infant mice.

**Summary.** MHV(PRI) virus produced a non-fatal immunizing infection in adult C<sub>3</sub>H mice over a greater than 6.0 log<sub>10</sub> unit range but a uniformly fatal infection in adult Princeton (PRI) mice. Neonatally thymectomized 4–6 week old C<sub>3</sub>H mice died by day 10 after inoculation with MHV(PRI). Intact and thymectomized C<sub>3</sub>H mice had comparable virus titers in their livers until day 7 postinfection after which time virus was undetectable in intact C<sub>3</sub>H mice but remained at high titers in thymectomized C<sub>3</sub>H mice. The liver pathology was similar in both groups until day 6 post infection after which time resolving lesions were seen in livers of intact C<sub>3</sub>H mice whereas thymectomized C<sub>3</sub>H mice developed fulminant fatal hepatitis. In *in vitro* tests, the macrophages of the thymectomized C<sub>3</sub>H mice did not support growth of MHV(PRI) virus to any greater extent than the macrophages of nonthymectomized C<sub>3</sub>H mice.

Although infection with MHV(PRI) was fatal for both PRI and thymectomized C<sub>3</sub>H mice, the course of infection was much more rapid in PRI mice. C<sub>3</sub>Hss mice which are congenic with C<sub>3</sub>H mice but have macrophages which support growth of MHV(PRI) responded to MHV(PRI) infection with a rapidly fatal illness in the same way as PRI mice. These data suggest that macrophages resistant to viral multiplication and intact thymic function are both necessary for resistance to the lethal effects of MHV(PRI) virus.

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## Thymidine Kinase and DNA Polymerase Activity in Normal and Zinc Deficient Developing Rat Embryos (40279)

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pidly dividing cells, DNA synthesis is reduced by zinc deficiency (1-5). It has been noted that the severe teratogenesis resulting from maternal dietary zinc deficiency may arise as a consequence of impaired synthesis during fetal organogenesis (2), as seen in regenerating rat liver (6), tumor (7), and rat connective tissue (8) have indicated that the decrease in DNA synthesis as a result of zinc deficiency may be linked to the association of zinc with one or more of the dependent enzymes involved in DNA synthesis. The activity of two regulatory enzymes in DNA synthesis, thymidine kinase and DNA polymerase (9), was found to be reduced in zinc deficient rats (7, 8). Thymidine kinase is of particular importance since zinc deficiency produced a significant reduction in the activity of this enzyme in regenerating liver within 10 hr after partial hepatectomy, whereas DNA polymerase activity, thymidine synthesis, and protein synthesis were unaffected until some hours later (10). Reed and Dreosti and Hurley (11) found the activity of thymidine kinase to be significantly lower in embryos taken at 12 days of gestation from zinc deficient dams than in embryos from controls.

In the present study, the effect of zinc deficiency on the activity of DNA polymerase and thymidine kinase was investigated in 12-day embryos. In addition, since a previous report suggested that 12-day embryos are relatively less sensitive to zinc deficiency than are older embryos (12), the activity of both thymidine kinase and DNA polymerase was measured in 9, 10, and 12-day embryos from zinc deficient and control dams to determine whether zinc was acting similarly at early stages of gestation. Similar studies were also undertaken to test the effect of in vitro supplementation with various other divalent metal ions on the activity of DNA polymerase. Similar data regarding thymidine kinase have been reported previously (11).

**Materials and methods.** *Materials.* (Methyl-<sup>3</sup>H) thymidine (spec. act. 2 Ci/mM) and (methyl-<sup>3</sup>H) thymidine 5'-triphosphate (spec. act. 15 Ci/mM) were purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. All other chemicals were purchased from Sigma Chemical Company, St Louis, MO. Whatman DEAE cellulose (DE 23) filter paper circles were obtained from Reeve Angel, 9 Bridewell Place, Clifton, NJ.

*Animals and diets.* Virgin female Sprague-Dawley rats weighing 210 ± 10 g were bred overnight with stock fed males. On day zero of gestation, as determined by the presence of sperm in the vaginal smear, the animals were placed individually in stainless steel cages. The animals were fed a zinc deficient diet *ad libitum*, or a control diet *ad libitum*, or a control diet in amounts limited to the mean daily food intake of the deficient group (referred to as "restricted intake").

The zinc deficient diet contained less than 0.5 ppm zinc as measured by atomic absorption spectroscopy. The control diet was the same purified diet as the zinc deficient diet except that it was supplemented with zinc as zinc carbonate to a level of 100 µg/g. The composition of the diet has been described previously (13). In addition, all animals received vitamins in glucose three times per week.

*Collection of samples.* On day 9, 10, 11, or 12 of gestation, the animals were killed and embryos were removed by caesarean section. In order to obtain sufficient tissue for the enzyme assay it was necessary to pool litters of embryos. Five litters were pooled for each 9-day sample, three litters for each 10-day sample, two litters for each 11-day sample, one litter for each 12-day sample.

*Enzyme assays.* Pooled embryos were homogenized in 12 vol of chilled 0.25 N Tris-HCl buffer, pH 8.0, and an enzyme solution was prepared for use in the subsequent assays as described by Witschi (14).



Thymidine kinase was assayed by a modified procedure described by Witschi (14). The reaction mixture contained in a final volume of 0.5 ml, 0.25 *N* Tris-HCl buffer (pH 8.0), 5.5  $\mu$ M ATP, 6.6  $\mu$ M 3-phosphoglyceric acid, 5.5  $\mu$ M  $MgCl_2$  and 2.5  $\mu$ M (5.0  $\mu$ Ci) (methyl- $^3H$ ) thymidine and 0.1 ml of the enzyme extract. The reaction mixture was incubated at 37° for 15 min and the reaction was stopped by immersing the assay tubes in boiling water for 1 min. After cooling and centrifugation at 1000g for 10 min, 50  $\mu$ l aliquots of the protein-free supernatants were spotted onto DEAE cellulose filter paper discs and the papers were washed in 1.0 *mM* ammonium formate, water, and 95% ethanol. Radioactivity on the dried paper discs was measured in a Nuclear Chicago Mark I liquid scintillation spectrophotometer.

DNA polymerase was determined by a modified procedure described by Witschi (14) and Lehman *et al.* (15). The reaction mixture contained in a final volume of 0.5 ml, 0.25 *N* Tris-HCl buffer (pH 8.0), 0.05  $\mu$ M d-ATP, 0.05  $\mu$ M d-CTP, 0.05  $\mu$ M d-GTP, 1.5  $\mu$ M  $MgCl_2$ , 1.5  $\mu$ M KCl, 0.05  $\mu$ M 2-mercaptoethanol, 50  $\mu$ g heat denatured DNA (70° for 15 min), 0.05  $\mu$ M (5  $\mu$ Ci) dTTP and 0.1 ml of the enzyme extract. After incubation at 37° for 1 hr, the reaction was stopped by the addition of 0.1 ml cold 1.0 *M*  $HClO_4$ . The precipitate was washed twice with 0.5 *M*  $HClO_4$ , dissolved in 0.3 *M* KOH (3 ml), incubated for 60 min at 37°, reprecipitated with cold 0.5 *M*  $HClO_4$ , and washed once more. The pellet was dissolved in 1 *M* NaOH

(2 ml) and 0.5 ml aliquots were withdrawn for radioactivity determinations.

**Metal ion supplementation.** In certain DNA polymerase assays, supplementary zinc and other metal ions (0.01–0.2 *mM*) were added to the incubation mixture before addition of  $^3H$ -dTTP. All metal salts used were spectrophotometrically pure and, except for the zinc salt, contained less than 0.05  $\mu$ g zinc/g.

**Protein assay.** The concentration of protein in the fetal homogenates was determined by the method of Lowry *et al.* (16).

**Statistical analysis.** Mean  $\pm$  SEM are reported. The statistical significance of differences between means was tested by Student's "t" test.

**Results.** The activity of thymidine kinase was significantly lower in 9, 10, 11, and 12 days embryos taken from females fed a zinc deficient diet than in embryos from either *ad libitum* fed ( $P < 0.05$ ) or restricted intake ( $P < 0.05$ ) controls (Table I). However, the percentage decrease in activity in the zinc deficient animals when compared with restricted intake controls was not as great in early embryos as in the 12-day embryos. In addition, the activity of thymidine kinase increased with increasing age of embryos in all three dietary groups (Table I). The percentage increase in activity was greatest at early stages of gestation. Activity in the 9-day groups was only twice that of background values.

DNA polymerase activity was also significantly lower in 9, 10, 11, and 12 day embryos from dams fed the zinc deficient diet than in embryos from either the *ad libitum* fed ( $P <$

TABLE I. EFFECT OF ZINC DEFICIENCY AND DAY OF GESTATION ON ACTIVITY OF THYMIDINE KINASE IN RAT EMBRYOS.<sup>a</sup>

Day of gestation	Groups					
	Control <i>ad libitum</i>		Control restricted intake		Zinc deficient	
	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)
9	79 $\pm$ 9**		81 $\pm$ 14**		64 $\pm$ 6*..	79
10	323 $\pm$ 66**	309	351 $\pm$ 52**	333	196 $\pm$ 32*..	206
11	665 $\pm$ 95	105	659 $\pm$ 101**	88	372 $\pm$ 76*	90
12***	729 $\pm$ 101	10	950 $\pm$ 48	44	356 $\pm$ 79*	—
						37

<sup>a</sup> Thymidine kinase activity expressed as pM  $^3H$ -thymidine incorporated/mg protein/hr.

\*  $P < 0.05$  compared to *ad libitum* and restricted intake controls.

\*\*  $P < 0.05$  compared to activity in 1-day older embryos in the same group.

\*\*\* Data from Dreosti, I. E., and Hurley, L. S., Proc. Soc. Exp. Biol. Med. 150, 161 (1975).

0.01) or restricted intake ( $P < 0.01$ ) controls (Table II). The percentage decrease in the zinc deficient groups when compared with restricted intake controls was similar at all 4 days of gestation. DNA polymerase activity also increased with increasing age of embryos in all three dietary groups, but the percentage daily increase was not as great as that found with thymidine kinase (Table II). Even at 9 days of gestation, embryos had appreciable levels of DNA polymerase activity.

Addition of zinc, as zinc chloride, to the assay medium (at levels between 0.01 mM and 0.05 mM) had little effect on the activity of DNA polymerase in extracts from zinc deficient and control embryos at 12 days of gestation (Table III). However, supplementation of these extracts with a higher level, 0.2 mM zinc, resulted in a statistically significant depression of activity in extracts from both zinc deficient and control embryos (19% and 21%, respectively).

In a further experiment (Table IV), addition of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Fe}^{2+}$  had no effect on the activity of DNA polymerase when added to the medium at concentrations of 0.01 mM or 0.2 mM.

**Discussion.** The low activity of thymidine kinase and DNA polymerase in embryos from zinc deficient dams confirms previous reports of reduced activity of these enzymes in zinc deficient mammalian tissues (6-8). It further suggests that impaired DNA synthesis and teratogenesis associated with zinc deficiency may be related to reduced activity of these enzymes during organogenesis.

The thymidine kinase salvage pathway is

important for DNA synthesis only in rapidly dividing cells, not in normal adult cells where the *de novo* pathway of DNA synthesis is predominant (9, 17). Therefore, the thymidine kinase pathway may be of critical importance in the developing embryo. Since the effect of zinc deficiency on cell division is most manifest in rapidly proliferating tissues, it is reasonable to suppose that thymidine kinase may be involved. In contrast, while the activity of certain DNA polymerase enzymes is enhanced in rapidly dividing cells, these enzymes, unlike thymidine kinase, are also important in DNA synthesis in normal resting cells (18, 19).

Further support for the idea that thymidine kinase, and possibly DNA polymerase, are possibly primary sites of action of zinc in embryonic tissue is provided by the finding of decreased activity of both enzymes with decreasing age of embryos. Hurley *et al.* (12) have found a low incidence of congenital

TABLE III. EFFECT OF SUPPLEMENTARY ZINC ON THE ACTIVITY OF DNA POLYMERASE IN 12-DAY RAT EMBRYOS.<sup>a</sup>

Zinc added (mM)	Percent of original DNA polymerase activity	
	Zinc supplemented control	Zinc deficient
—	100 ( $\pm 3.5$ )	100 ( $\pm 3.2$ )
0.01	106 ( $\pm 2.7$ )	113 ( $\pm 4.5$ )
0.05	114 ( $\pm 6.3$ )	121 ( $\pm 7.3$ )
0.2	79 ( $\pm 3.7$ )*	81 ( $\pm 3.1$ )*

<sup>a</sup> DNA polymerase activity expressed as nM  $^3\text{H}$ -TTP incorporated/mg protein/hr.

\*  $P < 0.05$  compared to extracts with no zinc added.

TABLE II. EFFECT ON ZINC DEFICIENCY AND DAY OF GESTATION ON ACTIVITY OF DNA POLYMERASE IN RAT EMBRYOS.<sup>a</sup>

Day of gestation	Groups					
	Control <i>ad libitum</i>		Control restricted intake		Zinc deficient	
	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	Activity	% of control restricted intake
9	2.32 $\pm$ 0.12**		2.19 $\pm$ 0.24**		1.44 $\pm$ 0.30* **	67
10	2.68 $\pm$ 0.25**	16	2.51 $\pm$ 0.22	15	1.62 $\pm$ 0.25* **	66
11	2.96 $\pm$ 0.28	10	2.62 $\pm$ 0.42	4	1.88 $\pm$ 0.25*	72
12	3.06 $\pm$ 0.35	3	2.59 $\pm$ 0.38		1.97 $\pm$ 0.26*	76

<sup>a</sup> DNA polymerase activity expressed as nM  $^3\text{H}$ -TTP incorporated/mg protein/hr.

\*  $P < 0.01$  compared to *ad libitum* and restricted intake controls.

\*\*  $P < 0.05$  compared to activity in 1-day older embryos in the same group.

TABLE IV. EFFECT OF SUPPLEMENTARY METAL IONS ON THE ACTIVITY OF DNA POLYMERASE IN 12-DAY EMBRYOS FROM ZINC DEFICIENT DAMS.<sup>a</sup>

Metal ion added	Concentration (mM)	Percent of original DNA polymerase activity
—	—	100 (±3.5)
Cu <sup>2+</sup>	0.01	92 (±3.6)
	0.2	85 (±6.8)
Cd <sup>2+</sup>	0.01	95 (±4.9)
	0.2	90 (±2.8)
Mn <sup>2+</sup>	0.01	98 (±3.7)
	0.2	98 (±5.7)
Mg <sup>2+</sup>	0.01	100 (±8.0)
	0.2	99 (±4.7)
Co <sup>2+</sup>	0.01	100 (±5.8)
	0.2	96 (±3.9)
Fe <sup>2+</sup>	0.01	97 (±9.1)
	0.2	93 (±4.0)

<sup>a</sup> DNA polymerase activity expressed as nM <sup>3</sup>H-TTP incorporated/mg protein/hr.

abnormalities in rats fed a zinc deficient diet from days 0 to 8 of pregnancy. The incidence of malformations increased when the animals were fed a zinc deficient diet for longer periods during gestation or for the same length of time but at a later stage of gestation. The very low activity of thymidine kinase in rat embryos at 9 days of gestation, together with the relatively smaller decrease in enzyme activity in 9-day embryos than in 12-day embryos from zinc deficient animals, may therefore make the early embryo relatively less sensitive to zinc deficiency than are later embryonic stages.

The failure of zinc added at the time of assay to restore the activity of DNA polymerase in zinc deficient enzyme extracts confirms earlier observations with extracts from regenerating rat liver (10) and suggests that zinc may not be associated with the enzyme as a readily dissociable cofactor. This finding is similar to observations with thymidine kinase and may be explained by a lack of incorporation of zinc into the enzyme at the time of synthesis. The possibility of reduced synthesis of the enzyme as a result of general reduced protein synthesis in the zinc deficient animals is unlikely since it has been shown in regenerating rat liver that protein synthesis was not affected by zinc deficiency until 10–20 hr after a change in DNA polymerase activity was noted (10).

The inhibitory effect of a high level of zinc (0.2 mM) on DNA polymerase activity *in*

*vitro* was similar to that reported by Dreosti and Hurley (11) for thymidine kinase. Such inhibition of activity of these two enzymes may account for the reduced DNA synthesis produced by high levels of zinc in cultured rat lymphocytes (20) and transplanted rat tumors (21).

Unlike thymidine kinase, which was relatively sensitive to Cd<sup>2+</sup> and Cu<sup>2+</sup>, addition of various metal ions at both low (0.01 mM) and high (0.2 mM) concentrations had little effect on the activity of DNA polymerase *in vitro*. This observation supports the data of Springgate *et al.* (22) using a zinc free apoenzyme and suggests that DNA polymerase is specifically zinc dependent.

In conclusion, the findings reported here indicate that the teratogenic effects of zinc deficiency in rats may arise from impaired activity of fetal thymidine kinase and DNA polymerase after day 8 of gestation and that the primary effect may be on the regulatory enzyme, thymidine kinase. The *in vitro* addition of metal ions to zinc deficient enzyme extracts suggests that zinc may not be associated with DNA polymerase as a readily dissociable cofactor and that DNA polymerase is specifically zinc dependent.

**Summary.** Thymidine kinase and DNA polymerase activities were significantly ( $P < 0.05$  and  $P < 0.01$ , respectively) lower in 9, 10, 11, and 12-day embryos taken from dams fed a zinc deficient diet than in those from *ad libitum* fed and restricted intake controls. An additional finding was that of increased activity of both thymidine kinase and DNA polymerase with increasing age of embryos. As previously found with thymidine kinase, addition of zinc and other divalent metal ions *in vitro* had little effect on restoration of DNA polymerase activity from zinc deficient extracts when added at concentrations of 0.01 and 0.05 mM. When added at a level of 0.2 mM, zinc, but not other metal ions, had an inhibitory effect on DNA polymerase activity. These findings support the hypothesis that the teratogenic effects of zinc deficiency are associated with the enzymes involved in DNA synthesis.

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L-Histidine-Induced Hypercholesterolemia: Characteristics of Cholesterol Biosynthesis in Rat Livers<sup>1</sup> (40280)JIRAPA K. SOLOMON AND RONALD L. GEISON<sup>2</sup>*Biomedical Research Unit, Waisman Center, and the Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706*

Dietary enrichment with high levels of single amino acids induced decreased food intake and growth suppression in young animals (1). The effects depend upon the kind and concentration of amino acid supplemented. Waisman and his colleagues have described in Rhesus monkeys a marked hyperlipemia associated with the dietary administration of L-histidine (2, 3). Histidine was the only amino acid of nine studied which induced this hyperlipemia. The hyperlipemia involved all circulating lipids. Serum phospholipids increased twofold, cholesterol two to threefold and triglycerides three to eightfold. Geison and Waisman fed 5% and 8% excess L-histidine diets to rabbits for 4 weeks and found a 50% increase in the plasma cholesterol level (4). Phospholipid levels did not change. The effect in rabbits was less pronounced than that observed in monkeys.

Our report presents the effect of dietary L-histidine supplementation in rats. We observe alterations in the incorporation of [2-<sup>14</sup>C] acetate or [1-<sup>14</sup>C] octanoate into lipids, studied in liver slices taken from rats fed a diet supplemented 5% with L-histidine.

**Materials and methods.** The basic diet fed in all experiments was Purina Formulab Chow containing 23% protein, 6.5% fat, 0.58% histidine, carbohydrate, vitamins and minerals. L-histidine (free base) was purchased from Ajinomoto, Co., Tokyo. [2-<sup>14</sup>C] acetate (specific activity 53.3 mCi/mole), [1-<sup>14</sup>C] octanoate (specific activity 3.5 mCi/mole), *Aquasol* (scintillation solution) and *Protosol* were purchased from New England Nuclear

Corp., Elmhurst, IL. DNA standard (Salmon testes) was purchased from Chemical Co., St. Louis, MO. Diphenylamine reagent was obtained from Allied Chemical, Palatine, IL. It was purified by recrystallizing from boiling hexane to obtain a crystalline product. Bovine serum albumin (Nutritional Biochemicals, Cleveland, OH) was used as protein standard. For homogenization, a motor-driven Potter-Elvehjemogenizer or a *Polytron* (Brinkman Instruments) was used. Liver slices were made with a McIlwain tissue chopper (The Micklethorpe Engineering Co., England). Incubation of liver slices was performed in a shaking Metabolic Shaking Incubator. All activity measurements were obtained with a Nuclear Chicago scintillation counter (model 300).

Male albino rats from Holtzman Rats, Madison, WI, were obtained at 21 days of age and weighed  $55 \pm 5$  g. They were housed in individual wire-bottom cages with a light-dark cycle changing every three hours throughout all experiments. Rats in the control group were fed ground Purina Formulab Chow *ad lib*. In the histidine-treated group, L-histidine constituted 5% of the diet by weight. It was added to the ground chow and fed to the rats *ad lib*. Since histidine-treated rats eat less than untreated controls, a second control group (pair-fed controls) was used. This group was fed the amount of food eaten by the histidine-treated group. After 4 days of feeding, the rats were killed by decapitation. Livers were isolated and either homogenized with 9 vol of distilled water for DNA and protein determination or sliced for the *in vitro* experiments.

Liver protein was estimated by the method of Lowry *et al.* (5) using bovine serum albumin as standard. DNA estimation was using the method of Schneider (6) with a DNA standard from Salmon testes.

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bation of liver slices with [2-<sup>14</sup>C] acetate [1-<sup>14</sup>C] octanoate was done under conditions suggested by Dietschy and Fry (7). CO<sub>2</sub> released was trapped by 20% in a cup hanging above the incubation mixture and the radioactivity was determined by dropping the cup into a scintillation counter containing Aquasol. Liver slices were washed with 0.9% NaCl three times and homogenized in distilled water using the Polytron. The homogenate was extracted with form-methanol in a 2:1 ratio. The extract was dried and dissolved in a small volume of chloroform, then spotted on a silica gel plate (0.25 mm thick). The plate was developed in a solvent system which contained heptane-ether-acetic acid in a 75:25:5 ratio. Lipid fractions were visualized by spraying the plate with 0.1% 2',7'-dichlorofluorescein in methanol. Each band was assayed for radioactivity and the rate of synthesis is expressed as nanomoles of the labeled precursor incorporated into the product per gram of liver per hour. The percent deviation from control in each fraction was calculated. All statistical analysis was done by using the paired Student's *t* test.

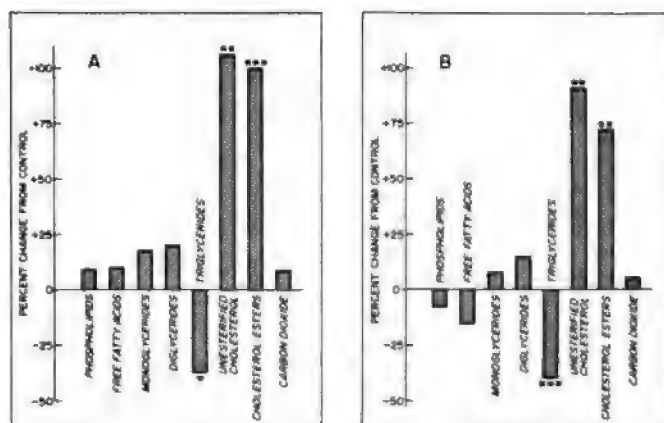
**Results.** [2-<sup>14</sup>C] Acetate and [1-<sup>14</sup>C] octanoate were found to incorporate into every fraction of liver lipids and the released CO<sub>2</sub> at various rates. L-histidine primarily affected the incorporation of the labeled substrates into cholesterol and triglycerides. When [2-<sup>14</sup>C] acetate was used as substrate, the in-

crease in its incorporation compared to controls was found to be 107% for unesterified cholesterol and 100% for cholesterol esters (Fig. 1-A). Both increases were statistically significant,  $P < 0.02$  and  $P < 0.01$  respectively. The opposite effect was observed in the case of triglycerides, which showed a significant ( $P < 0.05$ ) decrease of 36%.

When [2-<sup>14</sup>C] octanoate was used as substrate, there were significant ( $P < 0.02$ ) increases in the incorporation of the labeled substrate of 90% and 71% for unesterified cholesterol and cholesterol esters, respectively, compared to controls (Fig. 1-B). The incorporation into triglycerides was significantly ( $P < 0.01$ ) decreased by 39%.

Histidine did not significantly alter the incorporation of the labeled substrates into other liver lipids such as phospholipids, free fatty acids, monoglycerides and diglycerides. Histidine did not alter the activity of the tricarboxylic acid cycle, as indicated by the insignificant change of the incorporation of the labeled substrates into released CO<sub>2</sub>.

The effects of excess dietary L-histidine on cell size as estimated by liver DNA and protein contents are shown in Table I. The DNA to protein ratio in histidine-treated rat livers was significantly lower than the ratio observed in both *ad lib.* ( $P < 0.05$ ) and pair-fed ( $P < 0.01$ ) controls. The decrease in the ratio was 19.4% compared to pair-fed controls. Liver DNA and protein contents in histidine-treated rats were significantly ( $P < 0.01$ )



1. Percent change from control of the incorporation of [<sup>14</sup>C] acetate (A) and [<sup>14</sup>C] octanoate (B) into liver lipid fractions and CO<sub>2</sub> by liver due to L-histidine supplementation. The conditions of the incubation and separation of the lipid fractions are described in the methods. Asterisks indicate *P* values for comparison with *ad lib.* controls: \* $P < 0.05$ , \*\* $P < 0.02$  and \*\*\* $P < 0.01$ .

TABLE I. EFFECT OF 5% DIETARY L-HISTIDINE SUPPLEMENTATION ON DNA AND PROTEIN CONTENTS LIVER.<sup>a</sup>

Diet	DNA (mg/100 g liver)	Protein (g/100 g liver)	$\frac{\text{DNA}}{\text{protein}} \times$
AD LIB: 95% Chow + 5% L-Histidine (5)	147.7 $\pm$ 11.9 <sup>b</sup>	18.3 $\pm$ 0.5 <sup>b</sup>	8.0 $\pm$ 0
AD LIB: Chow (5)	166.0 $\pm$ 3.3	17.1 $\pm$ 0.6	9.8 $\pm$ 0
Pair-Fed: Chow (5)	224.5 $\pm$ 3.0 <sup>c</sup>	22.6 $\pm$ 0.5 <sup>c</sup>	10.0 $\pm$ 0

<sup>a</sup> Results are expressed as mean  $\pm$  S.E.M.<sup>b</sup> Significantly different from pair-fed controls,  $P < 0.01$ .<sup>c</sup> Significantly different from *ad lib.* controls,  $P < 0.001$ .<sup>d</sup> Significantly different from *ad lib.* controls,  $P < 0.05$ .<sup>e</sup> (N) = number of rats per group.

lower than levels in the pair-fed controls.

**Discussion.** L-histidine induces in young rats a hypercholesterolemia which occurs after a brief period of feeding (4 days). Histidine-treated rats are smaller than controls, have larger livers and 30–40% higher levels of plasma cholesterol (8). In the present study, the incorporation of [2-<sup>14</sup>C]acetate into cholesterol by liver was found to increase by 100% with the feeding of an L-histidine enriched diet. However, Dietschy and McGarry (7) have shown that the acetyl-CoA available for cholesterol synthesis in the cytosol is not in isotopic equilibrium with the intramitochondrial pool. In order to verify the result, [1-<sup>14</sup>C] octanoate was used as substrate under the same conditions. Octanoate is incorporated into cholesterol by the cytosolic biosynthesis pathway only after its intramitochondrial oxidation to acetyl-CoA. In this way the C<sub>2</sub> units entering the cholesterol biosynthetic pathway were in isotopic equilibrium with the intramitochondrial C<sub>2</sub> pool. Increases in the incorporation of the [1-<sup>14</sup>C] octanoate into unesterified cholesterol and cholesterol esters by 90% and 71%, respectively, were observed.

The second significant effect of histidine on liver in this study was the 36–39% decrease in the incorporation of the labeled substrates into triglycerides. Kerr *et al.* (3) showed that histidine-induced hyperlipemia in monkeys was easily detected by the appearance of a "creamy" serum reflecting the predominant presence of triglyceride-laden chylomicrons. The decrease in triglyceride synthesis observed in our experiments was in accord with the absence of "creamy" serum in the rat.

Dietschy and McGarry have shown the concentrations of the labeled substrates used in these experiments (4 mM for acetate and

1.1 mM for octanoate) to be saturating the metabolic process under study (7) also have reported that octanoate was efficient precursor than acetate for sterol synthesis. This contrasts with the data obtained in the present study where we observed in liver the rate of cholesterol synthesis with [2-<sup>14</sup>C] acetate was not significantly different from that obtained with [1-<sup>14</sup>C] octanoate.

Changes in the liver DNA to protein ratio support an increase in hepatic cell size which correlates well with previous results showing a 100% increase in liver glycogen content. In summary, the present study demonstrates that dietary enrichment with L-histidine induces specific effects in cholesterol and triglyceride synthesis in weanling rats. These effects might represent the regulation of specific enzymes in cholesterol biosynthesis and lipogenesis such as  $\beta$ -hydroxy- $\beta$ -methylglutaryl coenzyme A reductase and fatty acid synthetase.

**Summary.** A diet supplemented 5% L-histidine caused a 100% increase in the incorporation of [2-<sup>14</sup>C] acetate or [1-<sup>14</sup>C] octanoate into cholesterol in liver slices of weanling rats after four days of feeding. The incorporation of the labeled substrates into triglycerides decreased 38%. The hepatic DNA to protein ratio decreased 19% with histidine feeding, suggesting an increase in hepatic cell size.

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## Effect of Cholera Toxin on Renal Tubular Reabsorption of Glucose and Bicarbonate (40281)<sup>1</sup>

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Cholera toxin (CT) produces fluid and electrolyte secretion in the small intestine due to stimulation of adenylate cyclase and increased production of adenosine 3'5' cyclic monophosphate (cAMP) (1-6). Other studies have shown that the adenylate cyclase-cyclic AMP system is stimulated by CT in a variety of tissues such as liver, thyroid, adrenal, fat and leukocytes with no demonstration of any other major structural or enzymatic changes (7-13). Thus, CT may provide a pharmacologic tool for the study of the effects of stimulating adenylate cyclase-cyclic AMP systems (10, 11).

We have previously shown that the infusion of cholera toxin into one renal artery of dogs is followed by decreased net tubular reabsorption of sodium, potassium, calcium, magnesium and phosphate with interrelationships similar to those observed during expansion of the extracellular fluid volume with saline (14). Further studies from our laboratory have demonstrated that expansion of the extracellular fluid with a Ringer bicarbonate solution is accompanied by increased net production of cyclic AMP by the kidney suggesting a role for cyclic AMP in the reabsorption of these various ions (15).

Since extracellular fluid volume expansion is accompanied by decreased tubular reabsorption of glucose (16, 17) and bicarbonate (18-20) due to suppression in their reabsorption, which occurs mostly in the proximal tubule (20-25), this study was designed to evaluate whether the stimulation of renal adenylate cyclase with CT also affects the reabsorption of these two substances in an attempt to further document a relationship between renal cAMP and tubular reabsorptive processes.

*Material and methods.* Twelve experiments were carried out in female mongrel dogs weighing from 18 to 27 kg, anesthetized with pentobarbital (30 mg/kg). The dogs were ventilated through a cuffed endotrachea with a Harvard Respirator. Both ureters were cannulated through bilateral flank incisions and a curved 23 gauge needle was placed into the left renal artery in the direction of flow. Isotonic saline was infused in the renal artery at a rate of 1 ml per min throughout the studies. A catheter was placed in the aorta through a femoral artery to obtain blood samples and to measure arterial pressure with an aneroid manometer. All experiments were started at least 60 min after completion of surgery. Glomerular filtration rate (GFR) was measured using the clearance of exogenous creatinine with standard procedure and constant infusion technique. Urinary collections of 10 min duration were obtained throughout the studies with blood obtained at the midpoint of each period. After control periods purified cholera toxin (Schwarz-Mann, Orangeburg, NY) was added to the renal arterial infusion to a rate of 8 µg/min for 180 min.

The effect of CT on glucose reabsorption was evaluated in five dogs. An intravenous solution containing glucose (10-15%), sodium (23 mEq/l), potassium (10 mEq/l) and acetate (33 mEq/l) was given at a rate of 1 ml/min in order to attain a stable high level of blood glucose at the time of the measurement of the effect of CT on tubular transport of electrolytes which usually occurs 100-140 min after the administration of CT (14). Both arterial and urine samples were collected in 10 min intervals.

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<sup>2</sup> Dr. Tuma is a Fellow of the American Heart Association, Greater Los Angeles Affiliate.

test tubes and triplicate determinations of glucose were performed.

The effect of CT on bicarbonate reabsorption was studied in seven dogs. In order to raise the blood bicarbonate to a stable level of 33–37 mEq/l, the animals received pulse injections of bicarbonate 50–90 mEq at the beginning of the study and every 40 min thereafter and a constant infusion of a solution containing bicarbonate (240 mEq/l), sodium (263 mEq/l), potassium (10 mEq/l), and chloride (33 mEq/l) at a rate of 4 ml/min. The rate of respiration was adjusted by the Harvard Respirator to keep  $PCO_2$  stable around 40 mm Hg. Urine was collected anaerobically under mineral oil from the ureteral catheters and blood samples were obtained anaerobically in syringes containing heparin.

These protocols allowed us to compare tubular reabsorption of glucose (TRG) and bicarbonate ( $TRHCO_3$ ) by both kidneys when all variables other than the infusion of CT into one renal artery were equal.

The concentration of creatinine in the blood and urine samples were determined with Technicon autoanalyzer (Tarrytown, NY), sodium and potassium with Instrumentation Laboratory flame photometer (Lexington, MA), chloride with CMT 10 chloridometer (Radiometer, Copenhagen), glucose with Beckman glucose analyzer (Beckman Instruments Incorporated, Palo Alto, California) which utilizes glucose oxidase (26), and pH and  $PCO_2$  with a Radiometer acid base analyzer, Model BMS 3-PHM71 (Radiometer, Copenhagen). The concentration of bicarbonate in plasma and urine were calculated from the Henderson-Hasselbach equation utilizing the following factors: Solubility coefficient for  $CO_2$  in plasma and urine of 0.0301 and 0.0309, respectively; a  $pK$  of 6.10 for plasma and a  $pK$  for urine calculated from its ionic strength according to the formula,  $pK_a = 6.33 - 0.5 \sqrt{Na^+ + K^+}$  with the concentrations of Na and K given in equivalents per liter (27). Paired data analysis was used to evaluate the statistical significance of the results which are expressed as mean  $\pm$  SEM.

**Results.** *Effect of CT on glucose reabsorption (TRG).* The effect of the infusion of CT on GFR, fractional excretion of sodium ( $FE_{Na}$ ) and glucose reabsorption are given in

Table I and Fig. 1. There were no significant differences among these parameters between both kidneys prior to the infusion of glucose and CT. Renal TRG after 100–140 min of CT was  $80.1 \pm 20.2$  mg/min, a value significantly ( $P < .05$ ) lower than that observed for the contralateral kidney ( $98.7 \pm 20.7$  mg/min). Renal TRG per 100 ml GFR was  $254 \pm 32.7$  mg, a value significantly ( $P < .01$ ) lower than that observed in the opposite kidney ( $363 \pm 43.5$  mg per 100 ml GFR). The  $FE_{Na}$  increased significantly from both kidneys but it was markedly higher ( $P < .01$ ) from the kidney receiving CT ( $11.2 \pm 2.82\%$ ) than the contralateral kidney ( $4.62 \pm 1.42\%$ ).

The values for TRG per 100 ml GFR in all measurements made from both kidneys during the period of 100–140 min after the initiation of the infusion of CT and when filtered glucose ranged between 700–1900 mg per 100 ml GFR are shown in Fig. 1. For any given level of filtered glucose, TRG per 100 ml GFR was lower in the kidney infused with CT.

*Effect of CT on bicarbonate reabsorption.* The effects of CT infusion on GFR,  $FE_{Na}$ ,  $TRHCO_3$ /GFR and the urinary excretion of sodium, chloride and bicarbonate are given in Table II and Figs. 2 and 3. Again, there were no significant differences between these parameters prior to the infusion of bicarbonate and CT. Renal  $TRHCO_3$  after 100–140 min of CT was not different between both kidneys while  $TRHCO_3$ /GFR  $\times 100$  by the infused kidney was  $2.09 \pm .06$  mEq per 100 ml GFR, a value significantly lower ( $P < .01$ ) than that observed in the contralateral kidney ( $2.53 \pm .06$  mEq per 100 ml GFR). Figure 2 provides data on  $TRHCO_3$ /GFR for all measurements obtained during the maximal effect of CT and a filtered bicarbonate ranging between 2.8 to 4.1 mEq per 100 ml GFR. Again,  $TRHCO_3$ /GFR  $\times 100$  for any given level of filtered carbonate was lower under the effect of CT.

The  $FE_{Na}$  increased in both kidneys but was significantly higher ( $P < .01$ ) in the kidney receiving CT ( $15.9 \pm 0.74\%$ ) than that of the contralateral kidney ( $7.1 \pm .26\%$ ). The increments in urinary sodium in the CT kidney were due to both NaCl diuresis (40%) and  $NaHCO_3$  excretion (60%) while the ex-

TABLE 1. EFFECTS OF CHOLERA TOXIN ON RENAL TUBULAR REABSORPTION OF GLUCOSE.<sup>a</sup>

Experiment	$C_{cr}$ ml/min		$C_{Na}/C_{cr} \times 100$ %		PG mg/dl	TRG mg/min		TRG/ $C_{cr} \times 100$ mg	
	L	R	L	R		L	R	L	R
1. Control	14.5	19.8	0.06	0.04	161	23.0	32.0	161.0	161.0
CT + glucose	23.0	23.7	6.50	1.23	993	65.9	84.5	285.3	355.6
2. Control	24.6	26.1	0.50	0.70	142	35.0	48.0	142.0	142.0
CT + glucose	21.1	16.6	16.20	8.50	975	26.0	33.0	123.6	197.4
3. Control	39.4	39.6	0.14	0.19	156	61.0	61.5	156.0	156.0
CT + glucose	51.6	38.2	14.70	1.41	1027	149.4	161.1	288.0	423.0
4. Control	37.8	37.0	0.99	0.27	124	46.7	45.6	124.0	124.0
CT + glucose	24.2	24.5	9.50	6.20	1570	68.8	106.3	283.0	433.0
5. Control	38.8	36.0	0.73	0.67	164	63.6	58.9	164.0	164.0
CT + glucose	29.3	26.6	9.13	5.77	1553	90.3	108.7	292.0	406.0
Control, mean	31.0	31.7	0.48	0.37	149.4	45.9	49.2	149.4	149.4
SEM	4.95	3.75	0.17	0.13	7.38	7.70	5.27	7.38	7.38
CT + glucose, mean	29.8	25.9	11.20	4.62	1123.6	80.1	98.7	254.4	363.0
SEM	5.61	3.50	1.82	1.42	138.2	20.2	20.7	32.7	43.5
<i>P</i>									
L vs R									
Control		NS		NS			NS		NS
CT + glucose		NS		<0.01			<0.05		<0.01
<i>P</i>									
Control vs CT + glucose	NS	NS	<0.01	<0.05	<0.01				

<sup>a</sup> Each point represents the mean of three to five consecutive collections. The results obtained during cholera toxin (CT) and glucose infusion represent the mean of three to five consecutive 10 min collections during the maximum response to CT and stable high plasma glucose.  $C_{cr}$  = clearance of exogenous creatinine.  $C_{Na}/C_{cr} \times 100$  = fraction of filtered sodium excreted. PG = plasma glucose. TRG/ $C_{cr} \times 100$  = renal tubular reabsorption of glucose per 100 ml of glomerular filtration. L = left kidney infused with cholera toxin 8  $\mu$ g per min; R = right noninfused kidney; Control = collections obtained of prior to the infusion of cholera toxin and glucose. CT + glucose = collections obtained at peak effects of cholera toxin (100–180 min) and stable levels of high plasma glucose.

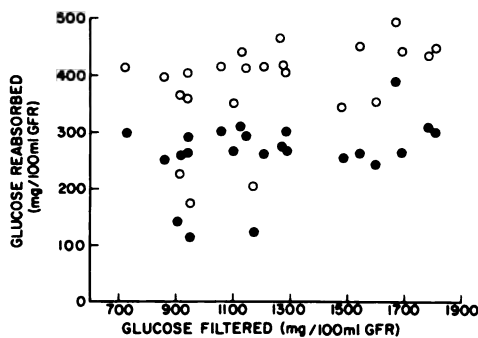


FIG. 1. The relationship between tubular reabsorption of glucose and filtered load of glucose in dogs receiving cholera toxin into the left renal artery. Data from the left kidney receiving cholera toxin infusion are shown in black dots and from the right kidney not receiving cholera toxin infusion are presented in open circles. Data is expressed as mg per 100 ml GFR.

cretion of NaCl comprised only 8% of urinary sodium from the contralateral kidney with the rest (92%) being  $\text{NaHCO}_3$  (Fig. 3).

**Discussion.** The results of the present study demonstrate that the infusion of CT into one renal artery is accompanied by a decrease in the renal tubular reabsorption of both glucose

and bicarbonate by the infused kidney.

Changes in glomerular filtration rate and alterations in status of extracellular fluid volume (ECF) are known to influence tubular reabsorption of glucose (16, 17, 20, 28, 29). Thus, when the absolute amount of glucose reabsorbed is plotted against GFR in animals in which the rates of sodium reabsorption were unchanged, a direct linear relationship was found (17). In our studies, GFR was either unchanged or modestly increased in the infused kidney at a time when TRG was lower. This observation clearly excludes changes in GFR as the cause for the reduced TRG by CT.

During expansion of ECF, there is an inverse relationship between tubular reabsorption of glucose per unit GFR and the fraction of filtered sodium excreted (17) suggesting that the mechanism responsible for the tubular reabsorption of glucose and sodium may be related. Furthermore, factors that inhibit renal transport of sodium such as ouabain or acetylcholinesterase suppress the reabsorption of glucose as well (30). Changes in the status of ECF could not account for

TABLE II. EFFECTS OF CHOLERA TOXIN ON RENAL TUBULAR REABSORPTION OF BICARBONATE.<sup>a</sup>

Experiment	$C_{Cr}$ ml/min		$C_{Na}/C_{Cr} \times 100$ %		PHCO <sub>2</sub> mmol/L	TRHCO <sub>2</sub> $\mu$ Eq/min		TRHCO <sub>2</sub> / $C_{Cr} \times 100$ mEq	
	L	R	L	R		L	R	L	R
1. Control	24.3	24.9	1.49	1.62	19.0	448	454	1.78	1.80
CT + NaHCO <sub>3</sub>	41.2	30.0	17.65	6.80	33.0	815	696	1.98	2.31
2. Control	45.8	46.4	0.10	0.09	23.2	1053	1070	2.29	2.31
CT + HCO <sub>3</sub>	25.4	26.1	17.40	6.54	34.2	496	646	1.96	2.48
3. Control	26.6	22.9	0.05	0.07	19.2	515	448	1.91	1.91
CT + HCO <sub>3</sub>	28.8	26.0	15.05	6.79	33.8	561	646	1.95	2.48
4. Control	32.2	32.1	0.27	0.31	22.5	719	717	2.24	2.24
CT + HCO <sub>3</sub>	40.1	34.0	15.97	6.63	35.2	912	873	2.27	2.56
5. Control	37.7	38.7	0.09	0.09	22.7	848	869	2.24	2.24
CT + HCO <sub>3</sub>	45.7	45.3	11.90	8.04	37.7	1028	1159	2.25	2.81
6. Control	37.0	36.0	2.21	2.20	19.6	714	695	1.93	1.93
CT + HCO <sub>3</sub>	33.1	27.1	16.85	8.17	33.2	659	651	1.98	2.40
7. Control	38.9	32.6	0.12	0.15	19.9	775	651	1.99	1.99
CT + HCO <sub>3</sub>	43.2	37.1	16.20	6.69	36.8	965	1001	2.24	2.69
Control, mean	34.6	33.4	0.62	0.65	20.9	724.6	700.6	2.05	2.05
SEM	2.82	3.04	0.33	0.33	0.69	76.6	83.4	0.08	0.08
CT + NaHCO <sub>3</sub> , mean	36.8	32.2	15.86	7.09	34.8	776.6	810.3	2.09	2.53
SEM	2.92	2.70	0.74	0.26	0.69	78.3	77.8	0.06	0.06
P									
L vs R									
Control	ND		NS			NS		NS	
CT + NaHCO <sub>3</sub>	<0.05		<0.01			NS		<0.01	
Control vs CT + NaHCO <sub>3</sub>									
	NS	NS	<0.01	<0.05	<0.01				

<sup>a</sup> Each point represents the mean of three to five consecutive collections. The results during cholera toxin and bicarbonate infusion represent the mean of three to five consecutive collections during the peak effect of cholera toxin (100-180 min) and stable high plasma bicarbonate.  $C_{Cr}$  = clearance of creatinine;  $C_{Na}/C_{Cr} \times 100$  = fraction of filtered sodium excreted; PHCO<sub>2</sub> = plasma bicarbonate; TRHCO<sub>2</sub> = tubular reabsorption of bicarbonate; TRHCO<sub>2</sub>/ $C_{Cr} \times 100$  = renal tubular reabsorption of bicarbonate per 100 ml of glomerular filtration; L = left kidney infused with cholera toxin, 8  $\mu$ g per min; R = right noninfused kidney; control = collections obtained prior to the infusion of cholera toxin and bicarbonate; CT + HCO<sub>3</sub> = collections obtained during maximum effect of cholera toxin and during stable high levels of serum bicarbonate.

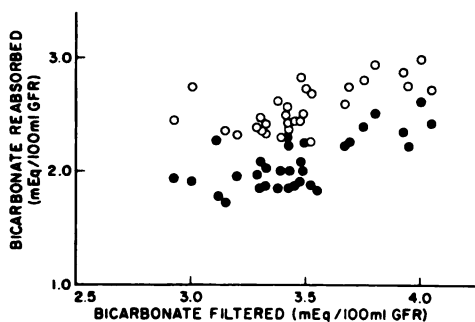


FIG. 2. The relationship between tubular reabsorption of bicarbonate and filtered load of bicarbonate in dogs receiving cholera toxin into the left renal artery. Data from the left kidney receiving cholera toxin infusion are shown in block dots and from the right kidney without infusion are presented in open circles. Data is expressed as mEq per 100 ml GFR.

our observation since both kidneys were subjected to the same conditions of ECF. However, CT produced a greater degree of natriuresis in the infused kidney and this may

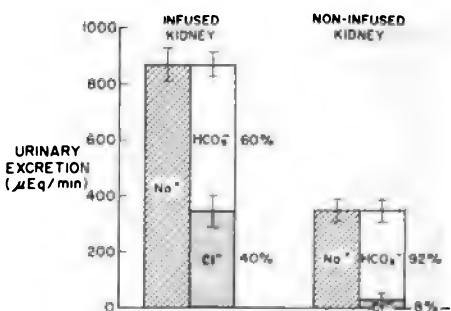


FIG. 3. Urinary excretions of sodium, bicarbonate and chloride during cholera toxin and bicarbonate infusion. Values are the mean and SEM of all experiments during maximal effects of cholera toxin.

directly or indirectly have influenced glucose reabsorption.

The tubular reabsorption of bicarbonate is affected by at least five factors. These include GFR (31), PCO<sub>2</sub> (32, 33), potassium metabolism (34), status of ECF (18, 19), and carbonic anhydrase activity (33). Most of these

factors could not account for the differences in  $\text{TRHCO}_3/\text{GFR}$  from both kidneys. Acute reductions in GFR are associated with a fall in absolute  $\text{TRHCO}_3$  but  $\text{TRHCO}_3/\text{GFR}$  remains unchanged (31), thus one would anticipate that a rise in GFR should be associated with a proportional increase in  $\text{TRHCO}_3$  with  $\text{TRHCO}_3/\text{GFR}$  remaining constant. In our bicarbonate infusion studies, the GFR in infused kidney was higher than the control kidney but  $\text{TRHCO}_3/\text{GFR}$  was lower. The blood levels of  $\text{PCO}_2$ , potassium, as well as the state of ECF could not provide explanation for the unilateral decrease in  $\text{TRHCO}_3/\text{GFR}$  since both kidneys were exposed to the same conditions.

The mechanism(s) through which CT affects glucose and bicarbonate reabsorption are not evident. Several possibilities should be considered. First, CT most probably affects renal tubular transport processes by the stimulation of a renal adenylate cyclase with increased production of cyclic AMP (14, 35). Several lines of evidence exist indicating that cyclic AMP reduces reabsorption of various ions in the proximal tubule (36–38). The studies of Lorentz (39) and Jacobson (46) suggest this effect of cAMP is mediated by an increase in tubular permeability allowing augmented back flux. It is therefore, plausible that the decrease in glucose and bicarbonate reabsorption during CT infusion is secondary to enhanced back flux of reabsorbate produced by cAMP. The observation of Karlin-sky *et al.* (41) who showed that the infusion of dibutyryl cyclic AMP reduced the tubular reabsorption of bicarbonate provides further support for the role of CT induced cAMP production in the genesis of reduced  $\text{TRHCO}_3$ .

Second, cholera toxin may directly affect the tubular transport of glucose and bicarbonate. In the ileum, CT enhances bicarbonate secretion (1) but there is no evidence for an effect of CT on glucose transport by the gut (42). Finally, CT may inhibit carbonic anhydrase activity and result in reduced reabsorption of bicarbonate; there is no evidence as yet supporting such a contention.

Most of glucose (21, 24, 25) and bicarbonate (22, 23) reabsorption occur in the proximal tubule. Our present observations of decreased TRG and  $\text{TRHCO}_3$  and natriuresis

are consistent with an effect of CT in the proximal tubule. However, marked decreases in proximal tubular reabsorption of sodium may not be followed by substantial natriuresis unless distal reabsorption of sodium is also reduced (43, 44). It seems, therefore, that CT should have an effect on tubular reabsorption in more distal portions of the nephron as well. Indeed, the observation that during bicarbonate loading 40% of the natriuresis in the infused kidney was due to NaCl as opposed to only 8% in the control noninfused kidney (Fig. 3) suggests that CT may have an effect on tubular reabsorption of Na at more distal sites of the nephron where Na is reabsorbed mostly as NaCl.

The present results together with our previous observations (14) have shown certain analogies between the natriuresis of expansion of ECF and that induced by CT: (a) Both are accompanied by depressed tubular reabsorption of glucose and bicarbonate, phosphate, calcium, magnesium and sodium chloride and (b) the relations between the fraction of filtered Na excreted and that of calcium and magnesium are similar in both conditions. Since extracellular fluid volume expansion with saline is accompanied by increased renal production of cyclic AMP (15) and the renal effects of CT are presumably mediated by stimulation of a renal adenylate cyclase-cyclic AMP system (14, 35), it could be postulated that at least part of the reduction in the tubular reabsorption of these various substances which occur during expansion of ECF may be mediated by increased production of cyclic AMP.

**Summary.** Cholera toxin (CT) reduces tubular reabsorption of Na, Cl, Ca, Mg and P most probably through stimulation of a renal adenylate cyclase-cyclic AMP system, and it is possible that an increased production of nephrogenous cyclic AMP during extracellular fluid volume expansion may be partly responsible for the observed natriuresis. In order to further evaluate the role of renal cyclic AMP in renal tubular transport, we studied the effect of CT on glucose (TRG) and bicarbonate reabsorption ( $\text{TRHCO}_3$ ).

During the period of maximal effect of CT on tubular transport (100–140 min of CT infusion into one renal artery) both the TRG and  $\text{TRHCO}_3$  were lower in the infused kid-

ney than in the contralateral noninfused kidney; TRG as mg per 100 ml GFR was  $254 \pm 32.7$  vs  $363 \pm 43.5$  ( $P < .01$ ), and  $\text{TRHCO}_3$  as mEq per 100 ml GFR was  $2.09 \pm 0.06$  vs  $2.53 \pm 0.06$  ( $P < .01$ ). The data indicate that CT suppresses glucose and bicarbonate reabsorption together with that of sodium and as such assign to role for renal cyclic AMP in the regulation of the tubular transport of these substances.

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## Shape Change and the Percentage of Sialic Acid Removed by Neuraminidase from Human Platelets<sup>1</sup> (40282)

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*N*-acetylneuraminic acid is the only sialic acid found in human platelets (1) and 7-15  $\mu$ g is found per mg of platelet protein (2). Bacterial neuraminidases from *Clostridium perfringens* and *Vibrio cholera*, which cleave the  $\alpha$ -ketoside linkage between sialic acid and the penultimate galactose or galactosamine, liberate 40-60% of sialic acid from human platelets (1-5). This is thought to be derived from surface membrane glycoproteins.

In a recent study, Motamed *et al.* (6) conclude that platelet surface sialic acid increases after shape change. This report is based on data obtained from platelets fixed in plasma. A nonspecific attachment of plasma proteins to the platelet surface is observed after fixation (7). As many of these plasma proteins contain sialic acid, and the platelet surface area increases after shape change (6), it is difficult to establish on the basis of these studies whether the increase in the amount of sialic acid removed by neuraminidase represents increased platelet surface sialic acid, or merely an increase in the amount of plasma proteins fixed onto the platelet membrane.

Ku and Wu (4, 5), using washed platelets, reported that thrombin-, collagen- and ADP-induced platelet activation increased the amount of neuraminidase-removable sialic acid. The increases observed after thrombin and collagen treatment were inhibited by aspirin, suggesting that material containing sialic acid is released during platelet activation, and that the increase in sialic acid observed after activation does not represent surface sialic acid.

This study was designed to determine whether ADP-induced shape change alters the amount of sialic acid on the surface of unfixed, aspirin-treated, gel-filtered platelets.

**Materials and methods.** *C. perfringens* neur-

aminidase (Type VI, Sigma) was purified according to the method of Hatton and Regoez (8) and shown to be free of proteolytic activity against radioactive tosylarginine methyl ester (TAME) (9) and fibrinogen (10). The activity of the purified enzyme was determined according to the method of Warren (11), and one unit is defined as the amount of enzyme required to liberate 1  $\mu$ mole of *N*-acetylneuraminic acid from bovine submaxillary mucin (Sigma) per min at 37°, pH 5.0.

Nine ml of blood from normal volunteers was collected into 1.5 ml of acid-citrate-dextrose (ACD) solution (12) and 0.5 ml of 1 mM acetylsalicylic acid (Merck) to inhibit the ADP-induced release reaction (13). After 15-min incubation at 37°, platelet-rich plasma (PRP) was prepared by centrifugation at 280g for 8 min at room temperature. PRP was removed and centrifuged at 2100g for 20 min. The resulting platelet button was resuspended in 1/2 the PRP volume with Tyrode's solution containing no added calcium (14), but with 0.2% bovine serum albumin (Sigma), and 0.1 mg/ml potato apyrase (Sigma purified Grade 1). The buffer was adjusted to pH 7.4 and the platelet concentrate gel-filtered (15) through Sepharose 2B (Pharmacia) equilibrated with Tyrode's solution containing albumin but no added glucose, calcium or apyrase. The platelets were eluted with the same buffer and their ability to aggregate with 5  $\mu$ M ADP was established on a small aliquot after adding 0.5 mg/ml fibrinogen (Kabi).

The suspension, containing  $0.5-1.0 \times 10^6$  platelets/ $\mu$ l, was divided into four 0.9 ml aliquots and brought to 37°. Two samples were treated with 0.1 ml 50  $\mu$ M ADP, and two with 0.1 ml 0.15 M NaCl. The tubes were gently inverted twice, and a small aliquot of each was fixed in 1% formalin for examination under the phase contrast microscope.

Approximately 1 min after the addition of ADP, the pH of the samples was adjusted to

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with 0.1 N HCl. One ADP-treated and saline control sample than received 40 neuraminidase (0.21 U/ml). The other treated and saline control sample received 40  $\mu$ l of ammonium acetate buffer. All samples were incubated for 15 min at 37°. After incubation, phase microscope observations showed that the saline control platelets retained their discoid shape and the suspensions exhibited the characteristic "swirl" when agitated. In contrast, the ADP-treated suspensions were spiny spheres and failed to swirl when shaken. A portion of the platelet suspensions not treated with neuraminidase was subjected to mild acid hydrolysis (0.1 N HCl, 1 hr, 80°) and total sialic acid was determined. Platelets were counted in another suspension with a Coulter Counter. The remaining portions of the control samples as well as the neuraminidase-treated samples were then centrifuged in a Serofuge (Clay Adams) for 10 min. The supernatants were removed and sialic acid measured without hydrolysis (11). In order to determine whether sialic acid is released from platelets after treatment with ADP and bin and connective tissue as reported by Wu (4), 9 ml of blood was collected into 1.5 ml of ACD containing 10  $\mu$ l of serotonin (Amersham, 8  $\mu$ Ci/ml) (16) and used as previously described. Fifty  $\mu$ l of a suspension of  $^{14}$ C-serotonin-labeled gel-d platelets was placed in glass counting vials containing 10 ml Aquasol (New England Nuclear) and counted in a Packard Liquid Scintillation Counter. Another aliquot, 0.5 ml, was used to determine total sialic acid without mild acid hydrolysis. The remaining suspension was divided into three equal aliquots and treated with 1 U/ml (final concentration) of highly purified human thrombin given to us by Dr. John W. Fenton, New York State Department of Health, Albany, a suspension of ground human subcutaneous connective tissue (given to us by Dr. J. Lackner, New York University Medical Center), or isotonic saline. After gentle mixing, the suspensions were incubated at 37° for 10 min. The samples were then chilled and centrifuged at 4°C at 2100 g for 20 min to pellet the platelets. The sialic acid was measured on 190  $\mu$ l of each supernatant after mild acid hydrolysis (11) and 50  $\mu$ l of each supernatant was used to measure  $^{14}$ C.

**Results.** The total sialic acid content of the ADP-treated and saline control samples did not differ. Hence, both values were included in the average, which was 60 nmoles/10<sup>9</sup> platelets (Table I). Neuraminidase removed 47% of sialic acid from both control and ADP-treated platelets. There was no sialic acid in the supernatants of samples treated with buffer instead of enzyme, even after hydrolysis.

Two experiments were carried out in which platelets were incubated with thrombin or connective tissue for 10 min without shaking. The platelets lost their "swirl" but no aggregates were seen on gross inspection.  $^{14}$ C-serotonin release in the saline control platelets was 13.6% and 4.2% (Table II). This material is presumably released when platelets come into contact with Sepharose beads during gel filtration (17). Platelets treated with thrombin released 89.1% and 81.2% of their  $^{14}$ C-serotonin, and 30% and 43% of their total sialic acid, respectively. Platelets treated with collagen released 57.6% and 33.2% of their  $^{14}$ C-serotonin, and 30% and 31% of their total sialic acid.

**Discussion.** ADP-induced shape change does not cause the release of material containing sialic acid from aspirin-treated platelets, as none was detected in the supernatants even after hydrolysis. Thus the sialic acid measured in the supernatant of suspensions treated with neuraminidase presumably represents sialic acid cleaved from membrane glycoproteins.

The amount of sialic acid removed by neuraminidase is not altered by ADP-induced shape change. This finding agrees with the results of Bunting and Zucker (3) who demonstrated that the same amount of tritium was incorporated into the sialic acid of discoid platelets and platelets which had undergone shape change after exposure to ADP.

Like others (2, 18), we found that collagen and thrombin release material containing sialic acid from human platelets. As neuraminidase will cleave sialic acid from both the platelet membrane and the released material, it is essential to prevent the release reaction in order to quantitate changes in membrane sialic acid during platelet stimulation.

Since we find no difference in the amount of sialic acid removed by neuraminidase from



TABLE I. THE EFFECT OF SHAPE CHANGE ON THE AMOUNT OF SIALIC ACID REMOVED BY NEURAMINIDASE FROM HUMAN PLATELETS.

Expt. No.	Total sialic acid (nmoles/10 <sup>9</sup> platelets)			Sialic acid removed by neuraminidase (%)		Change in sialic acid removed
	NaCl	ADP	Avg.	NaCl	ADP	
1	74	74	74	40	39	-1
2	90	91	90	40	40	0
3	58	58	58	42	42	0
4	50	46	48	64	66	+2
5	40	44	42	52	52	0
6	80	82	81	33	30	-3
7	45	50	47	59	63	+4
8	42	44	43	49	44	-5
Mean			60	47	47	-0.375 <sup>a</sup>
SE			0.6	0.8	0.8	0.98

<sup>a</sup> *t* = 0.382, not statistically significant.

TABLE II. PERCENT RELEASE OF <sup>14</sup>C SEROTONIN AND SIALIC ACID BY STIMULATED PLATELETS.

Expt. No.	Agent added	<sup>14</sup> C Release (% of Total)	Sialic acid in supernatant (% of Total)
1	NaCl	13.6	0
	Thrombin	89.1	30
	C.T.	57.6	30
2	NaCl	4.2	0
	Thrombin	81.2	43
	C.T.	33.2	31

discoid platelets and spiny spheres, we conclude that ADP-induced shape change does not alter platelet surface sialic acid. In contrast, sialic acid appears to be lost from platelets which have been aggregated with ADP and disaggregated (3).

**Summary.** The sialic acid of human gel-filtered platelets was studied before and after ADP-induced shape change. Neuraminidase cleaved 47% of the total sialic acid from both discoid control platelets and platelets that had become spiny spheres after treatment with 5  $\mu$ M ADP.

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# L-Histidine-Induced Facilitation of Cholesterol Biosynthesis in Rats<sup>1</sup> (40283)

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tritional loading of amino acids, particularly phenylalanine, has been used to study metabolic disorders. During such experimentation Waisman and his colleagues histidine-supplemented diets to infant monkeys and noted a marked hyperemia (1, 2). Later Geison and Waisman (3) 5% and 8% excess L-histidine diets to 4-old rabbits and induced a 50% increase plasma cholesterol levels. Our investigations have been pursued in rats, attempting to provide a more accessible animal model studying dietary histidine supplementa-

rats fed a diet supplemented 5% with L-histidine develop large livers and hypercholesterolemia (4). There is an increase in the incorporation of cholesterol precursors into cholesterol in liver slices from rats fed excess histidine (4). This finding prompted further investigation to determine the effect of histidine supplementation on cholesterol biosynthesis in the 5,000g supernatant solution of liver homogenate. Mature rats were used in this study because cholesterol and fatty acid metabolism in weanling rats is unstable, and the change of diet from milk to chow in previous studies have demonstrated that histidine decreases the rate of synthesis of cholesterol from acetate (6, 7). When fasted animals were refed a normal diet, the synthesis of cholesterol from acetate returned to normal within three days (8). When they were refed a fat-free diet, cholesterol synthesis declined to its normal level within three days and then declined to a very low level (8). This investigation studied the effects of histidine supplementation on the rate of synthesis of

cholesterol and cholesterol precursors from acetate and mevalonate in both normal and fat-free diets. All measurements were obtained during high and low diurnal levels of cholesterol synthesis.

**Materials and methods.** Experimental materials were obtained from the following sources: [2-<sup>14</sup>C] acetate (specific activity 53.3 mCi/mmole), [2-<sup>14</sup>C] RS-mevalonic acid, N,N'-dibenzylethylene diammonium salt (specific activity 40.2 mCi/mmole, and *Aquasol* (scintillation solution) from New England Nuclear Corp., Elmhurst, IL; glucose-6-phosphate, NAD, NADP, dithiothreitol, digitonin, and nicotinamide from Sigma Chemical Co., St. Louis, MO; EDTA from Fisher Scientific Co., Itasca, IL; L-histidine (free base) and bovine serum albumin from Nutritional Biochemical Corporation, Cleveland, OH. All other chemicals used were of analytical grade. The fat-free diet (Wooley and Seibell), Mod. TD-71125 was from Teklad Test Diets, Madison, WI. The normal diet was ground Purina Formulab Chow. In the histidine-supplemented diets, L-histidine constituted 5% of the diets by weight. A standard fitting Potter-Elvehjem homogenizer was used for homogenization. All radioactivity countings were done in a Nuclear Chicago Scintillation Counter, Isocap/300.

Male albino rats weighing 50-60 g each were obtained from Holtzman Rat Co., Madison, WI. Animals were divided into groups of four and fed normal and experimental diets *ad lib.* for 18 days after they were received. All rats, excluding the control group, then fasted for 2 days and were then refed experimental diets *ad lib.* for three days. This provided 21 days of experimental diet as used in previous studies of amino acid feeding (9). Rats were housed singly in stainless steel cages. The light cycle was from 7AM to 5:30PM.

**Preparation of rat liver homogenate.** Rats were sacrificed by decapitation, at 2PM or

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10PM, and the livers were removed quickly and placed on ice. Each liver was weighed, minced, and then homogenized in a 0.1 M potassium phosphate buffer, pH 7.4, containing 0.004 M  $\text{MgCl}_2$ , 0.001 M EDTA, and 0.002 M dithiothreitol, with five strokes of a Potter-Elvehjem homogenizer. The volume of buffer used was 2 ml/g of liver. The homogenate was centrifuged for 10 min at 5,000g. The volume of the supernatant solution was recorded. Protein concentrations were measured by a modification of the biuret procedure (10) using bovine serum albumin as standard.

**Assays for the conversion of acetate and mevalonate to NSF<sup>2</sup> and DPF<sup>3</sup>.** The rates of conversion of  $[2^{14}\text{C}]$  acetate and  $[2^{14}\text{C}]$  mevalonate to NSF and DPF were measured by a slight modification of the procedure of Slakey *et al.* (11). With acetate as the substrate, the incubation mixture contained 125  $\mu\text{l}$  (approximately 5.0 mg protein) of the 5,000g supernatant solution diluted to 0.5 ml with homogenizing buffer plus cofactors and  $[2^{14}\text{C}]$  acetate (2.5  $\mu\text{moles}$  and  $4 \times 10^5$  dpm per  $\mu\text{mole}$ ). With mevalonate as the substrate, the incubation mixture contained 75  $\mu\text{l}$  (approximately 3.0 mg protein) of the 5,000g supernatant solution diluted to 0.5 ml with homogenizing buffer plus cofactors and  $[2^{14}\text{C}]$  RS-mevalonate (2.5  $\mu\text{moles}$  and  $2 \times 10^5$  dpm per  $\mu\text{mole}$ ). The NSF was counted in a toluene scintillation solution and the DPF was counted in *Aquasol*.

**Results. Acetate to NSF and DPF.** The incorporation of  $[^{14}\text{C}]$  acetate into the NSF and DPF of the 5000g supernatant solution of rat liver homogenate is shown in Fig. 1. The labeled substrate was incorporated nine times more into the NSF of rats which were refed a histidine-supplemented chow diet than in those of the control group (Fig. 1A). This increase is statistically significant ( $P < 0.001$ ). Refeeding chow, fat-free, or a histidine-supplemented fat-free diet did not significantly affect the NSF synthesis activity. Refeeding of the histidine-supplemented chow diet induced a seven- to eightfold increase in the incorporation of the labeled

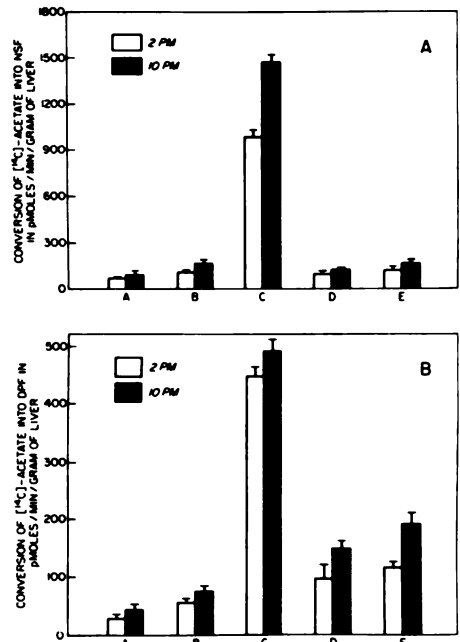


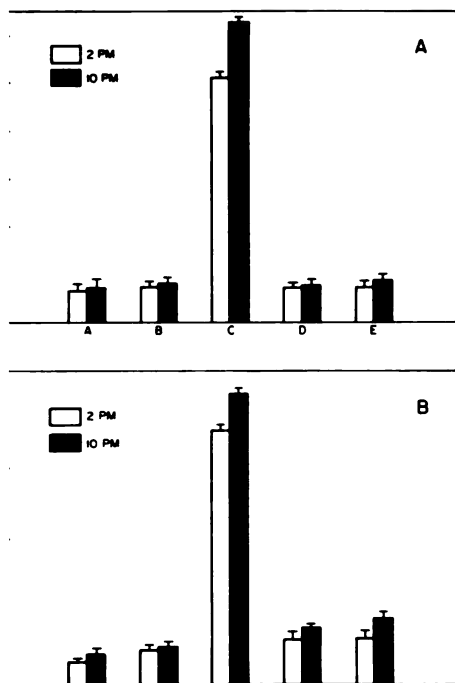
FIG. 1. Effects of L-histidine supplementation on the rate of conversion of  $[2^{14}\text{C}]$  acetate to the nonsaponifiable (A) and digitonin-precipitable (B) fractions in the 5000g supernatant solution of liver homogenates of rats maintained in different nutritional states: continuously fed, chow (A); fasted-refed, chow (B); fasted-refed, 95% chow + 5% L-histidine (C); fasted-refed, fat-free (D); and fasted-refed 95% fat-free + L-histidine (E). Vertical bars represent standard deviations with four rats in each group.

substrate into the DPF (Fig. 1B). This difference is also significant ( $P < 0.001$ ). Histidine supplementation to the fat-free diet did not cause a significant increase in the DPF synthesis activity.

**Mevalonate to NSF and DPF.** Effects of feeding excess histidine on the incorporation of  $[^{14}\text{C}]$  mevalonate into the NSF are shown in Fig. 2A. A 7.5-fold increase in total synthesis activity over the matched control was observed when refeeding the histidine-supplemented chow diet ( $P < 0.001$ ). Refeeding of chow, fat-free diet, and a histidine-supplemented fat-free diet did not significantly affect the NSF synthesis activity. The amount of  $[^{14}\text{C}]$  mevalonate incorporated into the DPF was seven times higher with the refed histidine-supplemented diet than with the refed chow diet ( $P < 0.001$ ), as shown in Fig. 2B. Synthesis activity was 1.6 times higher in

<sup>2</sup> NSF = Nonsaponifiable fraction: sterols, squalene, and terpenols.

<sup>3</sup> DPF = Digitonin-precipitable fraction: sterols.



2. Effects of L-histidine supplementation on the conversion of [2-<sup>14</sup>C]mevalonate to the non-precipitable (A) and digitonin-precipitable (B) fraction 5000g supernatant solution of liver homogenates maintained in different nutritional states defined in Fig. 1. Vertical bars represent standard deviation with four rats in each group.

which were refed the fat-free diet than in continuously fed control ( $P < 0.05$ ). Histidine supplementation to a fat-free diet did not cause any significant increase over the refed control.

**Discussion.** L-histidine or a histidine metabolite effectively stimulates sterol synthesis when added to chow diet. The marked increase (seven- to ninefold) in the incorporation rate of labeled substrates into both and DPF in this study is considerably greater than the increase in plasma cholesterol (30% over normal) which occurred in our histidine supplement study (4). A spontaneous increase in the degradation of sterol in the liver may be responsible for this disparity.

Cholesterol synthesis varies diurnally (9), however, none of the enzyme activity which converts mevalonate to squalene does. In this investigation, sterol synthesis from either acetate or mevalonate at the high

point of the day was 1.3–1.6 times greater than at the low point.

Under a variety of experimental conditions which reduce the conversion rate of acetate to cholesterol, the conversion rate of mevalonate to cholesterol does not change or changes much less dramatically than that of acetate (7, 14). However, in the case of stimulation of sterol biosynthesis by histidine, a similar rate increase was obtained when either acetate or mevalonate was used as the labeled substrate. The result suggests that histidine probably has a significant effect on an enzyme or enzymes in the synthesizing pathway between mevalonic acid and cholesterol. It will be interesting to investigate the activities of these enzymes in future studies.

Refeeding of either a chow or fat-free diet did not cause a marked change in sterol and squalene synthesis (1.2- to 1.8-fold increase over controls). This agrees with results obtained by Craig *et al.* (8) which show that the cholesterol synthesis activity rises from fasting levels to normal levels within three days after refeeding either chow or fat-free diet. Histidine supplementation to the fat-free diet did not cause a substantial change in the rate of sterol and squalene synthesis from acetate. This contrasts with the marked increase in sterol and squalene synthesis in the histidine-treated chow fed group.

The livers from rats fed fat-free diets, regardless of histidine treatment, were deep yellow due to fat accumulation. This probably resulted from a higher rate of fatty acid synthesis. If this is true, acetyl-CoA, a common precursor for these two divergent pathways (cholesterol and fatty acid synthesis), could be exhausted from an endogenous pool with long-term feeding, thus impeding histidine's stimulation of cholesterol synthesis from acetate in rats fed a fat-free diet. However, the conversion rate of mevalonate into sterols and squalene in rats which were fed a long-term fat-free diet also did not change when histidine was added to their diet. This result might not be anticipated if the absence of acetyl-CoA accounted solely for the lack of a histidine effect in rats fed a fat-free diet. The next step in the study of these processes will be to measure the actual activities of the specific enzymes, such as  $\beta$ -hydroxy- $\beta$ -methylglutaryl CoA reductase and fatty acid synthetase.

**Summary.** A diet supplemented 5% with L-histidine induces hypercholesterolemia in rats. To examine the mechanism involved, L-histidine was added to either a chow or fat-free diet and fed to rats for 18 days. After 2 days of fasting, the rats were refed the same diet for three days. There was a ninefold increase in the incorporation of [ $^{14}$ C] acetate into the nonsaponifiable fraction in the 5,000g hepatic fraction of histidine-supplemented chow-fed rats compared to controls. The increase in the incorporation of the labeled substrate into the digitonin-precipitable fraction was seven- to eightfold. The incorporation of [ $^{14}$ C]mevalonate was increased by sevenfold in both the nonsaponifiable and digitonin-precipitable fractions. Longterm histidine supplementation to fat-free diet did not affect the incorporation of either [ $^{14}$ C] acetate or [ $^{14}$ C] mevalonate into these fractions.

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# tion to *Clostridium botulinum* Cultures of Phage Controlling Type C Botulinum Toxin Production (40284)

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production by *Clostridium botulinum* and D is closely associated with phage infection. Cultures becomeogenic when cured of a specific temperate phage while nontoxigenic strains converted to toxigenicity when infected with phage (3-6, 8-10). However, the conversion does not occur with all combinations of phage and cultures; only certain culture pairings are productive of conversion. The needed specificity was explained; due to differences in the adsorption of phage to cells when three antigenic groups were identified among the converting phages. The possibility of other explanations has been suggested by a later report (6) which shows that the same culture can be made toxigenic or nontoxigenic by different phages.

In the present communication further experiments on the phage-culture specificity needed for toxigenicity conversion by comparing the conversion of one of the type C toxin-converting phages to several *C. botulinum* types D cultures and their nontoxigenic descendants.

**Materials and methods.** Table I shows the characteristics of the cultures used (9-11). Nontoxigenic (C)-A02 and (D)-139 can be lysogenized with c-st phage (from C-Stockholm) as to produce type C toxin.

Cultures were maintained in Bacto-Meat Medium (Difco Lab., Detroit, MI). For the tests, they were grown in LYG medium of pH 7.2 made of 1% lactalbumin (Nutritional Chemical Co., St. Louis, MO), 2% yeast extract (Difco), 0.5% glucose and 0.15% NaOH-HCl. Plating medium was Bacto-heart Infusion Agar (Difco) containing 10% whole human blood obtained from a blood bank. Plated cultures were incubated in glass jars but other cultures were in-

cubated in tightly closed screw-capped tubes. All cultures were incubated at 37°.

Filtrates of C-Stockholm cultured overnight in LYG contained c-st phage titer of  $10^{3-4}$  plaque forming units (pfu)/ml when plated with indicator culture (C)-A02. The phage was purified by three successive cycles of incubating a transferred plaque for 4 hr with (C)-A02 actively growing in 5 ml of LYG, filtering culture lysate through Millipore membrane of 450 nm pores, and replating.

Two ml of the broth culture from the third passage were added to 15 ml of a young culture ( $A_{520} = 0.2$ ) of (C)-A02 and the culture incubated until lysis occurred during the next 3-4 hr. The lysate, clarified by centrifugation and subsequent filtration through a Millipore membrane, had a titer of about  $10^7$  pfu/ml but the titer decreased during storage of more than one week at 4°. The titer was regained when the phage stock was treated as in the last passage used in its preparation.

Adsorption tests were done in T2 buffer made of 0.4% NaCl, 0.5%  $K_2SO_4$ , 0.15%  $KH_2PO_4$ , 0.3%  $Na_2HPO_4$ , 1 mM  $MgSO_4$ , 0.1 mM  $CaCl_2$ , and 0.001% gelatin (7). This buffer was used because of convenience rather than superiority over other media. Preliminary tests have shown that c-st phage adsorbs to (C)-A02 equally well in systems using LYG, T2 buffer, or T2 buffer containing 40 µg tryptophane/ml.

Cells for adsorption tests were collected from overnight incubated cultures by centrifugation at 3000g for 10 min and washed three times with T2 buffer. Suspensions of  $1 \times 10^8$  cells/ml were made on the basis of counts made on a Petroff-Hausser counting chamber.

The phage preparation was diluted 1:10 with T2 buffer. After holding separately in an ice water bath for 5 min, 1.8 ml cell and 0.2 ml phage preparations were combined and held at 4°. After the desired adsorption

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time, the suspension was centrifuged for 10 min at 4° and 6000g. Unadsorbed phage was quantified by plating 0.1 ml of the decimal dilution series of the supernatant with the indicator strain.

The frequency of conversion was determined by examining isolated colonies. After cell-phage contact, the cells were collected by low speed centrifugation (1000g, 10 min) and plated to obtain isolated colonies. Of the colonies developing during 2 days incubation, 20 random picks were subcultured separately for 3 days in tubes of Cooked Meat Medium. The presence of type C botulinum toxin in these cultures were determined by challenging mice ip with 0.5 ml of culture fluid.

**Results.** Adsorption curves of c-st phage reacting with (C)-A02 were not different from those reported for most other phage systems. Phage adsorption depended on the multiplicity of infection (MOI): starting with  $4.0 \times 10^5$  pfu and  $1.8 \times 10^8$  cells/ml, 98% of phage was adsorbed in 10 min while 50% was adsorbed when the cell concentration was  $5.5 \times 10^6$ /ml.

Adsorption of c-st phage to cells of different cultures during 20 min contact at 4° is shown in Table II. Several controls showed the reduction in free phage was due to specific adsorption. As part of the first experiment of

TABLE II. ABSORPTION OF c-ST PHAGE TO TYPE C AND D STRAINS AND NONTOXIGENIC STRAINS DERIVED FROM TOXIGENIC PARENTS.  $1 \times 10^6$  CELLS/ml; FREE pfu AFTER 20 min CELL-PHAGE CONTACT AT 4°C.

Strain	pfu/ml of supernatant fluid		
	Expt. 1	Expt. 2	Expt. 3
No cells	$4.3 \times 10^5$	$1.8 \times 10^5$	$1.2 \times 10^5$
(C)-A02	$4.0 \times 10^3$	$6.0 \times 10^3$	$3.4 \times 10^3$
(C)-6813		$1.7 \times 10^5$	
(C)-6814		$1.5 \times 10^5$	
(C)-N71			$7.0 \times 10^3$
(D)-139		$2.8 \times 10^4$	
(D)-151	$1.4 \times 10^4$		
(D)-SA		$1.5 \times 10^4$	
C-Stockholm			$4.0 \times 10^3$
D-1873	$1.0 \times 10^4$		
(C)-A02(c-st)			$3.8 \times 10^3$
(D)-139(c-st)			$1.3 \times 10^4$

Table II, possible adsorption to a nonproteolytic *C. botulinum* type B culture (QC strain) and a type E (Morai strain) was examined. The respective titers of  $4.5 \times 10^5$  and  $4.0 \times 10^5$  pfu/ml after the adsorption treatments showed that c-st did not adsorb to these cells nor was it adversely affected by them. Phage inactivating factors were not produced by cells since titers of  $5.2 \times 10^5$  and  $4.4 \times 10^5$  pfu/ml, respectively, were found after treating the phage suspension with cell-free culture fluids of (D)-151 and (D)-1873.

Some quantitative differences were found in retesting the same cultures, but the conclusion can be drawn that c-st adsorbed to all cultures except (C)-6813 and (C)-6814. The phage adsorbed best to indicator strain (C)-A02, parent toxigenic C-Stockholm, and converted (C)-A02(c-st). The phage adsorbed to a slightly less degree to D toxin producer D-1873 and nontoxigenics derived from parents producing this type of toxin.

As reported previously (11), the phage lysed broth cultures of only (C)-A02 and (D)-139. When the lysates were subcultured in Cooked Meat Medium, type C toxin was formed (12). The phage produced plaques on lawns of these two cultures but not on those of others. However, by degrees of clearing of broth cultures and numbers of plaques formed, the phage was more overtly active against (C)-A02 than (D)-139.

(C)-A02 and (D)-139 differed also in their rates of conversion to toxigenicity (Table III); with optimum conditions of cell-phage con-

TABLE I. CULTURE STRAINS USED.

Strain <sup>a</sup>	Type toxin produced	Origin
C-Stockholm	C	wild type
D-1873	D	wild type
(C)-A02(c-st)	C	(C)-A02 infected with c-st phage
(D)-139(c-st)	C	(D)-139 infected with c-st phage
(C)-A02	—	AO <sup>b</sup> treatment of C-Stockholm
(C)-N71	—	NG <sup>b</sup> treatment of C-Stockholm
(C)-6813	—	spontaneously from wild type C-6813
(C)-6814	—	spontaneously from wild type C-6814
(D)-139	—	AO treatment of D-1873
(D)-151	—	AO treatment of D-1873
(D)-SA	—	spontaneously from wild type D-South African

<sup>a</sup> For toxigenics, letter indicate type of toxin produced; letter in ( ) indicates toxin type of parent from which nontoxigenic was derived.

<sup>b</sup> AO = acridine orange; NG = nitrosoguanidine.

II. CONVERSION RATES OF (C)-A02 AND (D)-STRAINS BY c-st PHAGE WITH OPTIMUM INCUBATION TIMES (4 hr) AND IN PRESENCE OF 2% NaCl.

NaCl	MOI <sup>a</sup>	Incubation min <sup>b</sup>	Toxic colonies among 20 tested
—	0.1	240	19
—	0.5	40	4
+	0.5	40	4
—	0.1	240	3
—	0.5	40	0
+	0.5	40	0
—	5.0	40	8
+	5.0	40	9

<sup>a</sup> multiplicity of infection.  
<sup>b</sup> cell contact time at 37°.

OI = 0.1, 4 hr), the conversion to toxicity of (C)-A02 was significantly higher than (D)-139. Raising MOI to 5.0 and the conversion rate for (D)-139. 2% NaCl in the adsorption system in an attempt to increase conversion to toxigenicity did not favor greater conversion of

converted isolates of the two culture produced approximately the same level (4 LD<sub>50</sub>/ml) of toxin. When nontoxigenic isolates from the first treatment were added to a second conversion test, the conversion rate of (D)-139 was again lower than (C)-A02.

It was made of the possibility that a converted strain (D)-139(c-st) might be modified form that could convert (D)-139 at a higher rate than the c-st phage added directly from C-Stockholm. The filtrate of an overnight incubated (c-st) culture was added to separate, growing cultures of (D)-139 and (C)-A02. After 4-hr cell-phage contact, the mixtures were plated and 20 resulting colonies were used for toxicity tests. None of the (D)-139 isolates produced toxin although 13 of 15 A02 subcultures had been converted to toxigenicity.

**Discussion.** (C)-A02 and (D)-139 were both converted to type C toxigenicity by c-st phage although the conversion frequency was significantly higher for (C)-A02. The difference in the conversion rates is related to the effective phage adsorption to (C)-A02 measured by comparative adsorption

results and lysis of broth cultures.

Several reasons are involved in only certain phage-cell pairings being productive of conversion to toxigenicity. Included are cases where the cells lack receptors for phage attachment. This situation is illustrated by (C)-6813 and (C)-6814 to which c-st phage did not adsorb.

Since c-st adsorbed to some extent to all other cultures used, the conversion or non-conversion of these cultures is not determined by phage adsorption only. (C)-N71 is already lysogenized by a nonconverting phage. Since this phage has the same host spectrum and antigenicity as c-st (11), its presence in the cells would confer immunity against the converting c-st phage. The result would be non-conversion to toxigenicity in spite of adsorption of c-st to the cells.

This nonconverting phage could not be demonstrated in the remaining cultures to which c-st phage adsorbs without converting to toxigenicity. It is possible that some of these cultures carry a defective phage that confers immunity against c-st phage; in others, host controlled restriction (1, 2) may be important in preventing conversion.

**Summary.** C-st phage which governs production of type C botulinum toxin was mixed at 4° with cells of *C. botulinum* type C and D cultures and nontoxigenics derived from them. The phage adsorbed to all three cultures producing type C toxin, the one type D toxin producer, 2 of 4 nontoxigenics from type C parents and the three nontoxigenics originating from type D toxin producers. The phage adsorbed to some cultures without converting to toxigenicity. The two nontoxigenic cultures which could be converted to toxigenicity differed in degrees of phage adsorption and conversion rates.

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## Suppression of Chemical (DEN) Carcinogenesis in SWR/J Mice by Goat Antibodies Against Endogenous Murine Leukemia Viruses<sup>1</sup> (40285)

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tion of spontaneous leukemia in mice has been successful following passivation with IgGs raised against endogenous ecotropic murine leukemia viruses (MuLVs), specifically the radiation leukemia virus (RadLV) (1, 2). Also, passive immunity against 3-methylcholanthrene-induced lymphomas in weanling C3H/f mice was achieved using anti-RadLV IgG. Price, et al. have shown that Fischer rat embryos are not transformed by MuLVs unless they are first infected with MuLVs, and that the requirement of viral adsorption and replication could be fulfilled by ecotropic (RLV) and xenotropic (AT124) MuLVs; inhibition of viral replication by specific antiviral antibodies effected blocked cell transformation (5). Thus it is that the expression of endogenous viruses is a major determining factor in the reactions following carcinogen treatment *in vivo* and *in vitro*.

The carcinogenic effect of nitrosamines in mice is well documented (6-8). Diethylnitrosamine (DEN) treatment of SWR/J mice resulted in a high incidence of lung carcinomas and adenocarcinomas (72% versus untreated controls) 29 weeks after treatment (9). Since endogenous MuLVs are present in inbred strains of mice generally in association with age (10) and upon chemical carcinogen treatment (11), we decided to test the SWR/J system as a model for determining the involvement, if any, of endogenous MuLVs in chemical carcinogenesis in

SWR/J mice lack both infectious ecotropic and xenotropic MuLVs but express the specific antigen (p30) in both spleens

and thymuses (10). In the following communication we report that lung-tumorigenesis induced by diethylnitrosamine (DEN) in SWR/J mice is significantly delayed by treatments with antiviral antibodies against both RadLV and AT124.

**Materials and methods. Antiviral antibodies.** Goat IgGs raised against RadLV (Pool #3 NIH C5682) and AT124 (Pool #1 NIH C4928) were obtained from the Laboratory of RNA Tumor Viruses, NCI, Bethesda, MD 20014. These IgG preparations had neutralizing antibody titers of 1:800-1:1600 based on 70-100% inhibition of 60-70 AKR-XC plaques or 50-60 MSV (AKR) foci on SC-1 cells (12).

**Mice and treatments.** Twenty 8-week-old female SWR/J mice were pre- and post-treated with each goat anti RadLV and goat anti-AT124 IgG, and DEN according to the following schedule:

Day 0	0.1 ml anti-	0.1 ml anti-
(7-week-old)	AT124 IgG	RadLV IgG
Day 4	0.1 ml anti-	0.15 ml anti-
	AT124 IgG	RadLV IgG
Day 7	0.1 ml anti-	0.15 ml anti-
(10 AM)	AT124 IgG	RadLV IgG
Day 7 (5 PM)	DEN (90 mg/kg)	DEN (90 mg/kg)
Day 10	0.1 ml anti-	0.2 ml anti-
	AT124 IgG	RadLV IgG
Day 14	0.1 ml anti-	0.2 ml anti-
	AT124 IgG	RadLV IgG

Two groups of twenty control mice each received only the DEN treatment. DEN was freshly prepared in triethanolamine (Eastman Kodak) at a concentration of 10 mg/ml on each day of treatment. For each intraperitoneal injection the dose was 90 mg/kg (9).

The mice were set aside for tumor development and killed only when moribund. At necropsy lung nodules were counted and the lungs weighed to obtain a measure of tumor sizes. Lungs together with all other visceral organs were processed for histopathological evaluation according to standard procedures.

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While these experiments were under way, another group of 15 SWR/J mice received normal goat IgGs (Microbiological Associates, Bethesda) and DEN as per schedule used for anti-RadLV IgGs.

**Statistical evaluation of data.** All graphics and statistical analyses were done on a Tektronix microcomputer (Model 4051). Means, standard errors (S.E.) and analysis of variance were done according to Winner (13). *F*-tests were performed with 95% confidence intervals. The observed latency periods of AT124- and RadLV-IgGs treated groups of mice were compared with the corresponding untreated controls. The data on the normal goat IgG-treated group of mice was compared separately with all the other groups.

**Results and discussion.** DEN treated SWR/J mice passively immunized with goat anti-AT124 IgG survived up to 60 weeks following carcinogen treatment. Fifteen of 20 treated mice died from histologically confirmed lung tumors. In this group, 50% mortality because of lung tumors occurred at 54 weeks post-treatment. Thirteen of 20 untreated control mice died with multiple lung tumors and 32 weeks after treatment and the 50% mortality occurred by the 29th week (Fig. 1); seven mice had pneumonia.

Anti RadLV IgG immunized mice survived up to 64 weeks after DEN treatment. Eighteen of 20 mice developed histologically confirmed lung tumors by 64 weeks, with the

50% mortality incidence from lung occurring at 58 weeks. Control mice of this group survived only to 38 weeks with (12/20) lung tumor incidence (Fig. 2) (1). Five mice in this group died prematurely by a water bottle accident and the remaining three suffered from pneumonia. Since three mice receiving normal goat serum died from injection accidents.

Eight of 12 normal goat IgG treated

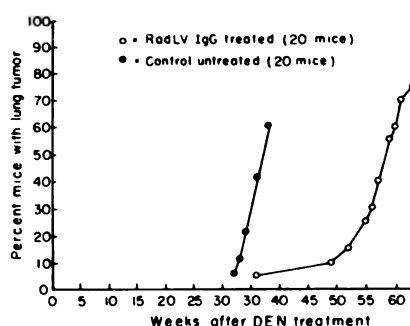


FIG. 2. Eight week-old SWR/J mice were given multiple doses of anti RadLV goat IgG and DEN (see Materials and Methods for details). Control mice received only DEN treatments. Anti RadLV IgG mice (○—○) showed a significantly prolonged latency period (Table I) when compared to untreated control mice (●—●).

TABLE I. EFFECTS OF ANTIVIRAL ANTIBODY ON LUNG TUMORIGENESIS IN DEN TREATED SWR/J MICE

Immunization <sup>a</sup>	Lung tumor incidence	Mean survival period in weeks (±SE) <sup>b</sup>
None	13/20	29.8 (±1.83)
Anti-AT124 goat IgG	15/20	52.0 (±1.70)
None	12/20	35.7 (±1.57)
Anti-RadLV goat IgG	18/20	57.33 (±1.28)
Normal goat IgG	8/12	34.87 (±2.23)

<sup>a</sup> Procedure for immunization is given in Materials and methods.

<sup>b</sup> Standard error in parenthesis.

<sup>c</sup> *F*-tests were done according to Winner (10).

<sup>d</sup> *F*α critical value.

<sup>e</sup> *F* value in comparison with untreated controls.

<sup>f</sup> Level of significance <0.0001 when compared with untreated controls.

<sup>g</sup> Normal IgG treated group when compared with other groups; the *F*-values for the different groups were: 38.46 (AT124 IgG), 3.15 (untreated control), 0.047 (RadLV IgG) and 0.047 (untreated controls).

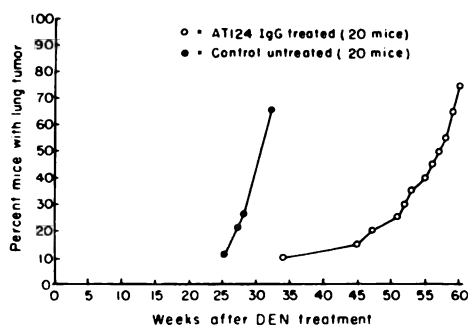


FIG. 1. Eight week-old SWR/J mice were pre- and posttreated with anti AT124 goat IgG and DEN as per schedule (see Materials and Methods). The mice were sacrificed when moribund and lungs and other viscera processed for histologic evaluation. Anti AT124 IgG treated mice (○—○) exhibited statistically significant (Table I) prolongation of their survival times when compared to untreated controls (●—●).

oped lung tumors by 40 weeks after treatment. The mean survival period significantly different from antiviral IgG and groups.

The distribution pattern of lung weights of treated and untreated mice is depicted in Fig. 3. Although immunized mice died with tumors after a long latency period, they had larger lung tumors (Fig. 3); obviously, tumors had more time to grow than in control mice which died early. All lung tumors were either alveolar adenomas or adenocarcinomas as described previously (9).

Indeed the presence of endogenous viruses is required for *in vivo* cell transformation by chemical carcinogens virus suppression should either prevent or prolong the onset of chemical carcinogenesis. The present data clearly indicate a very significant prolongation of the survival period of DEN-treated mice probably because of a slowed tumor growth in the antiviral IgG treated mice (Fig. 4). Antiviral IgG treated mice lived to an expected average life-span of 35 weeks SWR/J mice (9). Normal goat IgG is of beneficial effect on the survival time of DEN-treated SWR/J mice.

The data on lung tumor weights suggest that the antiviral IgGs had apparently very

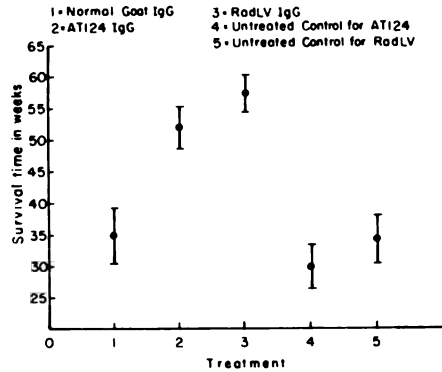


FIG. 4. Effect of antiviral IgGs on the latency period of DEN-induced lung tumors in SWR/J mice. The mean survival times after carcinogen was plotted against each treatment group. The bars (—●—) represent limits of variation. Both AT124- and RadLV IgG treated groups reveal a longer tumor latency period when compared to the control groups.

little effect, if any, on the growth rate of the tumors. Obviously by the time tumors appeared in most mice, the heterologous goat IgGs had long been eliminated.

Goat antiserum against MuLV gp71 as well as FeLV was shown to prevent oncornavirus induced sarcomas in cats (14). Thus it seems that the effectiveness of anti AT124 as well as RadLV IgGs against DEN carcinogenesis in SWR/J mice might be explained by the major homology between the two classes of MuLVs. Although the mechanism of the observed suppression of carcinogenesis is not clearly established, the data presented here tend to definitely indicate a viral involvement in chemical carcinogenesis. Presumably the antiviral IgGs partly suppress and delay the manifestations of chemical lung carcinogenesis in SWR/J mice.

**Summary.** We pre- and posttreated SWR/J mice given 90 mg/kg of DEN with goat anti RadLV and AT124 IgGs and studied their effects on the induction and latency of lung tumors. The results of these experiments tend to indicate a role of MuLVs in the etiology of the chemically induced lung tumors of SWR/J mice. The rate of tumor occurrence was greatly reduced in IgG treated mice and their survival time was significantly prolonged over nontreated mice. These findings require consideration of both ecotropic and xenotropic virus classes or their structural protein as cofactors in the chemi-

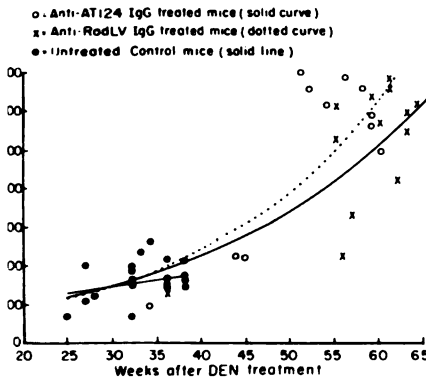


FIG. 3. Eight week-old SWR/J mice were given anti AT124 or anti RadLV IgG (see Materials and Methods details) and DEN. Control mice received only DEN. Wet lung weights of these mice were obtained at autopsy. Antiviral IgG treated mice developed larger lung tumors after a significantly prolonged latency period. The lung-weight distribution curves for AT124 (solid curve), RadLV (dotted curve) and untreated control (solid line) were obtained and plotted using a computer (Tektronix-4051).

cally induced lung carcinogenesis process. Similar conclusions were drawn previously by others in an *in vitro* chemical transformation system (5).

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# Endotoxin Induced Metabolic Alterations in BCG Infected (Hyperreactive) Mice (40286)

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Endotoxin given to laboratory animals causes a loss of glucose homeostasis which is characterized by hypoglycemia and death (1, 2). It has been accumulated to date suggest that endotoxin is a major factor responsible for hypoglycemia. The failure of gluconeogenesis rather than excessive glucose consumption, although the latter has been reported (3). Endotoxin induced hypoglycemia has been most extensively studied in mice by Berry and coworkers. These workers found that a key liver enzyme, phosphoenolpyruvate carboxykinase (PEPCK) which regulates gluconeogenesis is no longer susceptible to glucocorticoid inhibition after endotoxin poisoning (4, 6, 7). They have stressed the importance of this enzyme in endotoxin hypoglycemia presumably because this enzyme has a relatively short half life, i.e. 2 hr in rats while other gluconeogenic enzymes have longer half lives. The published data indicate that three gluconeogenic enzymes, glucose-6-phosphatase, glucose-1,6-diphosphatase and PEPCK, are at normal or elevated activities in endotoxin poisoned mice until 2 hr. Thereafter, the activities fall (8, 9), drop in enzyme activity corresponds to the development of hypoglycemia. A possible scenario for endotoxin induced hypoglycemia based on these observations is as follows: Mice given endotoxin fail to eat, and therefore, do not assimilate exogenous carbohydrates. Gluconeogenic enzymes are not induced by endogenous steroids elaborated in response to stress. As their level falls during enzyme turnover, particularly that of PEPCK, gluconeogenesis fails and hypoglycemia re-

sults. Mice often causes profound hypoglycemia in 2 hr, and death with convulsions frequently occurs within 4 hr (11). This is unlike the response of normal mice which die after 17-24 hr and in which the hypoglycemia occurs later and is less severe. The response of the BCG mouse is, therefore, a caricature of that of the normal mouse.

The exaggerated responses and shortened time course of BCG mice provide a suitable model to study metabolic abnormalities caused by endotoxin. We studied the BCG mouse model previously and found that endotoxin induced hypoglycemia is largely due to defective gluconeogenesis (2). Where in the gluconeogenic pathway the defect lies is unknown. In addition, the rapidity with which profound hypoglycemia occurs in BCG mice given endotoxin (2 hr vs 17 hr for normal mice) suggests that failure of enzyme induction and normal enzyme turnover may not account for this abnormality.

In this paper we report experiments designed to determine if the hypoglycemia in endotoxin poisoned BCG mice is due to a selective defect in the gluconeogenic pathway or if there is a general perturbation of the pathway. The studies were performed between one and 2 hr after endotoxin (prior to profound hypoglycemia) to avoid the potential secondary effects of hypoglycemia and shock. Additional studies were performed to determine the effect of glucocorticoid and stimulation of gluconeogenesis by fasting on endotoxin hypoglycemia and mortality.

**Materials and methods.** *Animals.* Pathogen free, CD-1 female mice weighing 20-25 g were obtained from Charles River Breeding Laboratories, North Wilmington, Massachusetts. They were fed and watered *ad libitum* and housed in air conditioned quarters fully accredited by the American Association of Laboratory Animal Care. Unless otherwise indicated, all animals were fasted 18-24 hr prior to experimentation. The mice were ren-

der infected with *Mycobacterium bovis* become hyperreactive to endotoxin and died approximately 1/1000th of the  $LD_{50}$  (10). These mice have a remarkable abbreviated clinical course with augmented clinical manifestations. One tenth of a gram of endotoxin in BCG infected

dered hyperreactive to endotoxin by a systemic infection with *Mycobacterium bovis* BCG given intravenously 13–16 days prior to use according to the method of Suter and Kirsanow (10). 0.2 ml of a 10–14 day culture of BCG in Dubos Liquid Broth (BBL) was injected via tail vein into unanesthetized, restrained mice.

**Endotoxin.** The endotoxin was prepared from a smooth strain of *Salmonella typhimurium*. The bacteria were grown in glucose minimal salts medium (M-9) supplemented with 0.1% Casamino Acids (Difco). At the stationary phase of growth they were killed with 0.2% formalin, harvested, and extracted by the phenol water procedure of Westphal *et al.* (12). Endotoxin challenge was by the intravenous route in 0.2 ml saline.

**Metabolic studies.** Glucose determinations were performed using the "Glucostat" (Worthington Biochemicals) micromethod. A 20  $\mu$ l sample of blood obtained from the retroorbital plexus was added to 1.0 ml distilled water and deproteinized with 0.5 ml 1.8%  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  and 0.5 ml 2.0%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  solutions. One ml of the resulting supernatant fluid was added to one ml "Glucostat" reagent at room temperature. A standard curve was prepared for each series of reactions.

Glucose production *in vivo* was estimated by the net increase in blood glucose twenty minutes following an intraperitoneal injection of 100  $\mu$ M glycerol or fructose. Endotoxin was given intravenously one hour before glycerol or fructose. In one experiment, the incorporation of  $^{14}\text{C}$  into glucose from  $^{14}\text{C}$  glycerol (3  $\mu\text{Ci}$  in 100  $\mu\text{M}$ ) was determined by measuring the cpm/mg glucose in blood obtained via cardiac puncture. The glucose was separated from 1.0 ml whole blood by passage through mixed bed resin columns as described by Corridor *et al.* (13). The effluent was qualitatively checked chromatographically to insure that the radiolabel resided with the glucose.

Substrate oxidation *in vivo* in mice was measured by methods previously described (2). The mice were adapted to a gas train in such a way that all expired air was bubbled through 5 ml  $\text{NCS}^{\text{TM}}$  (Nuclear Chicago Corp.) to collect  $\text{CO}_2$ . Aliquots (0.5 ml) were removed at 15-min intervals and counted in a

Packard liquid scintillation spectrometer to determine the activity of  $^{14}\text{CO}_2$ . amounts of 1- $^{14}\text{C}$  glycerol, 6- $^{14}\text{C}$  1- $^{14}\text{C}$  glucose, or 1- $^{14}\text{C}$  palmitate (New England Nuclear) were injected intravenously in control BCG infected mice and in BCG infected mice one hour after 1.0  $\mu\text{g}$  endotoxin. The isotopes (specific activities 4.6–6.0  $\mu\text{Ci}/\text{mmole}$ ) were injected in 0.2 cc saline vein.

The free fatty acid concentration in sera of individual mice was measured spectrophotometrically at 440 nm and compared with similarly treated standards of palmitic acid dissolved in chloroform. The free fatty acids were extracted from the sera by mixing 1 ml in 2.0 ml 0.2 M phosphate buffer (pH 7.4) and 6.0 ml chloroform. The mixture was shaken 2 min and after settling 15 min the upper layer was removed by aspiration. The chloroform layer was filtered into clean, dry, chloroform rinsed glass stoppered tubes. To 3.0 ml  $\text{Cu}$ -triethanolamine reagent was added and mixed. The color was developed by the addition of two drops of sodium diethyldithiocarbamate reagent before reading the absorbance at 440 nm (14).

**Results.** Previous experiments have shown that glucose production from pyruvate decreased in BCG infected mice as early as 2 hr after endotoxin challenge. The gluconeogenic pathway from pyruvate to glucose involves all of the key gluconeogenic enzymes including phosphoenolpyruvate carboxykinase. The pathways from fructose and glycerol do not. Therefore, if the endotoxin induced defect in gluconeogenesis is the loss of a specific enzyme at the beginning of the pathway one would expect glucose production from glycerol and fructose might be unaffected. The results presented in Tables I and II, however, indicate that the metabolic lesion is not limited to the loss of phosphoenolpyruvate carboxykinase since glucose production *in vivo* was maintained from glycerol or fructose. The incorporation of exogenous fructose and glycerol into blood glucose in the control mice, but not in BCG infected mice, prevented a decrease in blood glucose given to BCG infected mice one hour after endotoxin. In a similar experiment, labeled  $^{14}\text{C}$  glycerol was used to insure that an actual decrease occurred in the incorp-

TABLE I. THE EFFECT OF EXOGENOUS FRUCTOSE ON BLOOD GLUCOSE CONCENTRATION IN BCG INFECTED MICE BEFORE AND AFTER ENDOTOXIN.

Treatment	Mean blood glucose, mg percent $\pm$ SE	
	Before fructose <sup>a</sup>	20 min after fructose
BCG infected controls (10)	91 $\pm$ 3	109 $\pm$ 2
BCG infected mice 1 hr after 1.0 $\mu$ g endotoxin (10)	81 $\pm$ 9	65 $\pm$ 3

<sup>a</sup> 100  $\mu$ M fructose injected ip. ( ) Indicates the number of mice per group.

TABLE II. GLUCOSE PRODUCTION FROM GLYCEROL IN BCG INFECTED MICE BEFORE AND AFTER ENDOTOXIN.

Treatment	Mean blood glucose, mg percent $\pm$ SE		cpm/mg glucose $\times 10^{-4}$
	Before glycerol	20 min after glycerol	
BCG infected control mice	Exp 1		
	115 $\pm$ 6 (10) <sup>a</sup>	142 $\pm$ 5 (10)	
BCG infected mice 1 hr after 1 $\mu$ g endotoxin	Exp 2		
	111 $\pm$ 9 (10)	158 $\pm$ 9 (10)	9.6 $\pm$ 1 (10)
BCG infected mice 1 hr after 1 $\mu$ g endotoxin	Exp 1		
	76 $\pm$ 10 (10)	56 $\pm$ 5 (10)	
	Exp 2		
	91 $\pm$ 12 (10)	66 $\pm$ 18 (10)	3.6 $\pm$ 1 (10)

<sup>a</sup> ( ) Indicates the number of mice per group.

of  $^{14}\text{C}$  label into blood glucose. The results shown in Table II indicate that the incorporation of  $^{14}\text{C}$  into blood glucose in the experimental group was only about one-third the incorporation which occurred in the control mice not given endotoxin.

Because an increased oxidation of glycerol might account for its decreased incorporation into glucose, the *in vivo* oxidation of glycerol was measured between one and 2 hr after endotoxin by collecting expired  $^{14}\text{CO}_2$  after  $^{14}\text{C}$  glycerol injection. Figure 1 shows the cumulative counts per minute of  $^{14}\text{CO}_2$  collected from a group of BCG infected mice and a group of BCG mice given endotoxin.

The results show decreased glycerol oxidation after endotoxin.

Since an increased oxidation of glucose could result in an apparent decrease in incorporation of  $^{14}\text{C}$  into blood glucose by its loss as expired  $^{14}\text{CO}_2$ , the *in vivo* oxidation of 1- $^{14}\text{C}$ ]glucose and 6- $^{14}\text{C}$ ]glucose was measured in mice after endotoxin. The results presented in Fig. 2 show that endotoxin caused decreased oxidation of both 1- $^{14}\text{C}$ ]glucose or 6- $^{14}\text{C}$ ]glucose. Oxidation of the 6- $^{14}\text{C}$ ]glucose was depressed more than that of 1- $^{14}\text{C}$ ]glucose.

Endotoxin  $\text{LD}_{50}$ 's were determined in fasted and fed BCG infected mice to determine if the fasting state, which enhances gluconeogenesis via endogenous steroids, influenced survival after endotoxin challenge. Fasted mice would have a stimulated gluconeogenic pathway and little stored carbohydrate while fed mice would have less active gluconeogenesis and much stored carbohydrate. The results presented in Table III show no difference in the responses of fasted and fed mice.

The effect of treatment of mice pre- and postchallenge with pharmacologic doses of

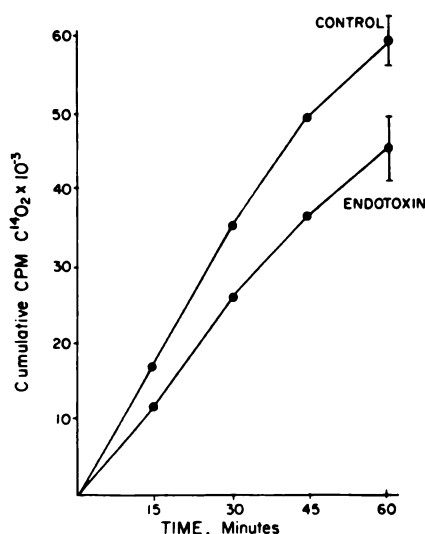


FIG. 1. Glycerol oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxin treated mice were given one  $\mu$ g endotoxin iv one hour prior to the injection of  $^{14}\text{C}$  glycerol. Each point represents the mean cumulative counts per minute of expired  $^{14}\text{CO}_2$  from five individual mice. Vertical bars indicate  $\pm$  SD.



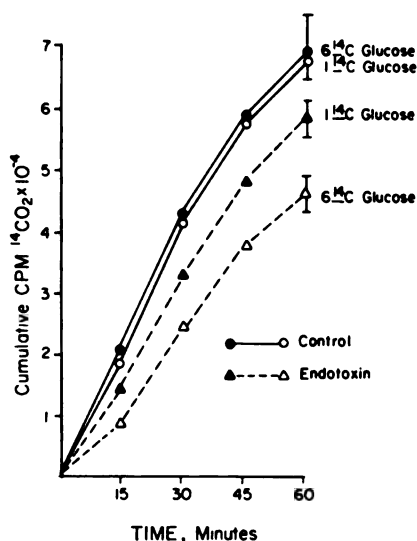


FIG. 2. Glucose oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxin treated mice were given one  $\mu\text{g}$  endotoxin iv 1 hr prior to the injection of [ $^{14}\text{C}$ ]glucose. Each point represents the mean cumulative counts per minute of expired  $^{14}\text{CO}_2$  from five individual mice. The vertical bars indicate  $\pm$  SD.

TABLE III. ENDOTOXIN  $\text{LD}_{50}$ 's IN FED AND FASTED BCG INFECTED MICE.

Dose Endotoxin ( $\mu\text{g}$ )	Fed		Fasted	
	dead/total	mean time to death (hours)	dead/total	mean time to death (hours)
3.2	5/5	5.5	4/5	5.6
0.8	5/5	4.8	4/5	4.9
0.2	4/5	5.3	4/5	7.0
0.05	1/5	—	1/5	—
$\text{LD}_{50}^a$	0.126 $\mu\text{g}$		0.163 $\mu\text{g}$	

<sup>a</sup>  $\text{LD}_{50}$ 's were obtained by the method of Reed and Muench (15).

hydrocortisone was studied to evaluate protection against endotoxin in BCG mice. The results presented in Fig. 3 show blood glucose concentrations and mortality after 1.0  $\mu\text{g}$  endotoxin when 3 mg hydrocortisone (Solu Cortef, Upjohn) was administered either before or after endotoxin. Cortisone reduced mortality significantly only in the group given cortisone prior to endotoxin. However, the rate at which blood glucose fell was diminished quickly i.e. within 2 hr, in all groups

receiving steroids. In a similar experiment, the mice were challenged with less endotoxin (0.1  $\mu\text{g}$ ). The results were similar except that mortality was also reduced in the group given cortisone 30 min after endotoxin. These experiments show that cortisone given before or after endotoxin challenge rapidly lessens the rate at which blood glucose falls and, depending on the timing, prolongs survival or prevents death.

Fatty acid oxidation is important in providing energy and reducing equivalents to drive the gluconeogenic pathway. Therefore, palmitate oxidation was measured in BCG infected mice and in similar mice between 1 and 2 hr after endotoxin challenge. The results shown in Fig. 4 show that the *in vivo* oxidation of palmitate was reduced about 50% in the endotoxin poisoned mice. This apparent reduction in palmitate oxidation, however may be due to an *in vivo* pool size difference. Table IV, showing the serum free fatty acid levels in BCG infected mice before and after endotoxin, indicates that endotoxin caused a 77% increase in circulating free fatty acids. The effective specific activity of the injected isotope would therefore be decreased in mice given endotoxin, and this could account for decreased  $^{14}\text{CO}_2$  evolution even though the rate of fatty acid oxidation is

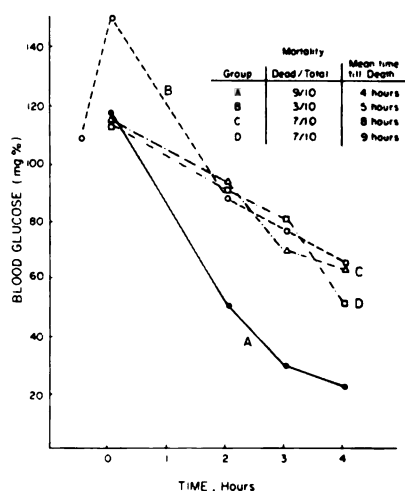
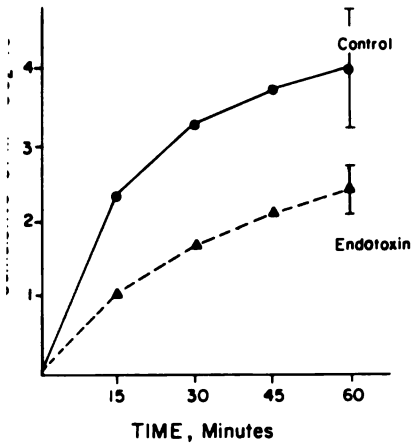


FIG. 3. Blood glucose concentration and mortality in BCG infected mice given 1.0  $\mu\text{g}$  endotoxin (A) and 3.0 mg hydrocortisone 30 min before endotoxin (B), and 30 min and 1 hr after endotoxin (C and D). Each point represents the mean blood glucose of 10 mice.



4. Palmitate oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxinated mice were given one  $\mu\text{g}$  endotoxin *iv* one hour prior to the injection of  $1\text{-}[^{14}\text{C}]\text{palmitate}$ . Each point represents the mean cumulative counts per minute of  $^{14}\text{CO}_2$  from five individual mice. The vertical bar indicates  $\pm$  SD.

ing. On the other hand, a similar explanation for the reduction in glucose oxidation is untenable since the concentration of glucose is less in the endotoxin poisoned BCG infected mice than in control mice not given endotoxin.

**Discussion.** The data in this paper show that endotoxin causes a general derangement of the gluconeogenic pathway in BCG-mice. Poisoned animals were unable to make glucose efficiently from glycerol or fructose. Previously we showed that glucose production from pyruvate was also impaired (2). Since these substrates enter the gluconeogenic pathway at different levels, a single lesion is unlikely to be responsible for the abnormality. In addition, the data also show that the present decreased incorporation of the substrate into glucose could not be caused by increased catabolism. The rates of oxidation of both glucose and glycerol were diminished. Corticosteroids in pharmacological doses protected BCG-mice from the lethal effect of endotoxin when preadministered. Even when given as long as 30 min after endotoxin, steroids exerted a rapid sparing effect on blood glucose. The rapidity with which this sparing effect occurred *i.e.* within 2 hr, raises the question as to whether the effect was by corticoid induced production of gluco-

neogenic enzymes. Increased enzyme production in response to glucocorticoids is a relatively slow process. The rise in enzyme is slow and usually preceded by a lag of 2–3 hr (16, 17). It seems more likely in this setting that the steroid was preventing some of the toxic effects of endotoxin and thereby lessening hypoglycemia or, alternatively, was activating gluconeogenic enzymes. This argument is also supported by the observation that protection against endotoxin requires pharmacological doses of corticosteroids while only physiological doses are sufficient for enzyme induction.

The study of fasted and fed mice given endotoxin also raises questions about the failure of enzyme induction by corticosteroids as a cause of hypoglycemia in BCG mice. Fasted animals have an active gluconeogenic pathway with elevated levels of gluconeogenic enzymes (7), while fed animals have high stores of carbohydrate but low gluconeogenic activity. When challenged with endotoxin fed animals rapidly deplete their glycogen stores and then have to depend on their low gluconeogenic activity. One might think that the fasted animal with high gluconeogenic activity might have the advantage in survival. However, in spite of this increase in gluconeogenesis, the outcome is the same. Stimulation of gluconeogenesis by endogenous physiological amounts of glucocorticoid, therefore, offers no protection.

The data also suggest that abnormal substrate oxidation may also be partially responsible for endotoxin induced hypoglycemia. The oxidation of fatty acids are required for the production of energy and reducing equivalents to drive the gluconeogenic pathway. Palmitate oxidation was diminished in BCG-mice given endotoxin. However, because of the increase in free fatty acids in the blood of

TABLE IV. THE EFFECT OF ENDOTOXIN ON SERUM FREE FATTY ACID LEVEL IN BCG INFECTED MICE.

Treatment	Free Fatty Acids ( $\mu\text{eq/ml}$ serum) $\pm$ SE
BCG infected control mice	$.97 \pm .03$ (14) <sup>a</sup>
BCG infected mice 2 hr after endotoxin	$1.72 \pm .03$ (15)

<sup>a</sup> ( ) Indicates the number of mice.

BCG-mice after endotoxin a correction has to be made for fatty acid pool size. Fatty acids increased about 77%. Palmitate oxidation decreased by about the same amount. The conclusion is that there was no real change in fatty acid oxidation. However, during normal homeostasis a profound fall in blood glucose should result in an increase in fatty acid oxidation. The failure of fatty acid oxidation to increase suggests that loss of homeostatic regulation after endotoxin includes lipid as well as carbohydrate metabolism.

**Summary.** The cause of hypoglycemia induced by endotoxin in BCG infected mice was investigated. The major abnormality, known to be defective gluconeogenesis, was studied to determine whether a specific point in the gluconeogenic pathway is involved or whether the derangement is more general. The inability of endotoxin poisoned mice to synthesize glucose from glycerol and fructose in addition to pyruvate indicated that the entire pathway was in disarray. The *in vivo* oxidation of glucose, glycerol and palmitate to CO<sub>2</sub> was reduced, indicating that enhanced aerobic oxidation was not responsible for the hypoglycemia. This decrease in substrate oxidation also suggests that impaired gluconeogenesis may be due to decreased energy available to maintain the gluconeogenic pathway. Pharmacologic doses of glucocorticoids were protective in endotoxin poisoned BCG infected mice. The rate of development of hypoglycemia was rapidly lessened, and mortality reduced. The data suggest that steroids confer protection by preventing or interfering with some of the toxic effects of endotoxin or perhaps by activating glyconeogenic en-

zymes. It is unlikely that glucocorticoid mediated enzyme induction plays an anti-endotoxin role in this model.

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# Granulocyte Mobility Induced by Chemotactic Factor in the Agarose Plate (40287)

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Keller *et al.* proposed a definition related to the locomotion of leukocytes (1). In their proposal, responses to chemotactic (and/or chemokinetic) factors are classified into two types, namely, chemokinesis and chemotaxis. Although the definition of these reactions is clear, it is not easy to separate these reactions separately in several kinds of methods, some evaluated chemokinesis and chemotaxis but it is not certain as yet whether these types of reactions can be recognized initially separated phenomena. In this study we analyzed chemokinesis and chemotaxis from chemotaxis in the agarose

plate. The purified granulocytes contained less than 1% of other cells including platelets.

**Chemotactic factor preparation:** Bacterial chemotactic factor was produced by overnight growth of *Escherichia coli* in heart infusion broth at 37°. The culture broth was passed through a 0.22 µm filter and the filtrate was then stored at -70° until use.

**Assay of leucocyte mobility.** This was performed by a minor modification of Nelson's method (6). The agarose plate was prepared by placing 3 ml of 1% agarose (Behringwerke) solution in Medium 199 containing 10% heat inactivated fetal calf serum into 35 mm × 35 mm Falcon plastic dishes. When chemokinesis, namely the enhancement of random mobility by chemotactic factor was assayed, the chemotactic factor was added uniformly to agarose. After the agarose gelled, 3 mm × 3 mm wells were made by a stainless steel punch in the agarose plate, and 10 µl of cell suspensions and chemotactic factor were placed as shown in Fig. 1. After various periods of incubation in a 5% CO<sub>2</sub> incubator at 37°, the distance the cells moved was measured under an inverted microscope with an ocular grid. Four measurements were averaged from the margin of the well to the line of migration in four quadrants of each well. All experiments were carried out in duplicate or triplicate.

**Preparation of cells for morphological examination.** After incubation, the cells were fixed with agarose in place by flooding the plates with 4 ml of 10% formalin for 48 hours. After fixation, the agarose was gently removed, and the cells were stained by Giemsa solution.

**Results. Random mobility and chemokinesis in the agarose plate.** The random mobility and chemokinesis of normal adult granulocytes assayed by the agarose plate method are shown in Table I. Granulocytes stimulated by 10% *E. coli*-derived chemotactic factor added uniformly in agarose showed a  $2.6 \pm$

**Materials and methods. Leucocyte preparation.** Adult blood was mixed with an equal volume of a 2% methyl cellulose solution (Kakai Chemicals, Japan) and was settled at room temperature for 30 min. Granulocytes in the supernatant were separated by centrifugation at 250g for 10 min and the cells were washed in ice-cold Hanks solution. The mononuclear cells were separated from the leucocyte preparation by a modification of the method of Boyum (2). The leucocyte suspensions in Hanks solution were layered on top of Ficoll (Pharmacia)-Hypaque (Winthrop) solution and centrifuged at 250g for 45 min at 4°. The mononuclear cell layer from the top of the gradient and the Ficoll-Hypaque solution were gently aspirated and discarded, and the cell button was resuspended in 0.2 ml of Hanks solution. Contaminating erythrocytes were disrupted by hypotonic shock. After washing twice with Hanks solution, the cells were resuspended in Medium 199 containing 10% heat inactivated fetal calf serum at  $1 \times 10^8$  cells per ml. For chemotactic assays, chemotactic factor was added to the medium at a final concentration

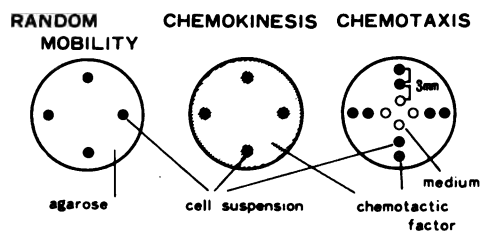


FIG. 1. Agarose plate method employed in the experiments. Three types of granulocyte mobility were measured in this method. The oblique lines means the presence of chemotactic factor derived from *E. coli*.

0.3 ( $M \pm SE$ ) fold enhanced mobility compared to nonstimulated granulocytes. Microscopic appearance of the preparations stained by Giemsa solution is shown in Figs. 2 and 3. Cells moving under the influence of chemotactic factor tend to be more irregular in outline than those moving without the influence of factor, and formation of blebs or pseudopodium-like structures can be observed. There is no regular orientation of cell axis.

Then we assayed the two types of granulocyte mobility as a function of time (Fig. 4). Cells under the influence of chemotactic factor moved rapidly up to 3 h, after which time no marked increase in distance was observed. On the other hand, in the absence of the bacterial factor movement of granulocytes increased linearly. However, even after 19 hours these cells had not moved as far as those stimulated by chemotactic factor.

*Relationship between the concentrations of chemotactic factor and the degree of chemo-*

*kinesis.* As shown in Fig. 5, granulocyte mobility increased linearly in proportion to concentration of chemotactic factor. More than 2.5% of chemotactic factor stimulation of migration diminished further with more than 10% of chemotactic factor, granulocyte mobility tended to decrease, although a significantly enhanced mobility could be observed when compared against random mobility.

*Granulocyte mobility under a concentration gradient of chemotactic factor.* First we examined the influence of a negative concentration gradient. In order to form a concentration gradient of chemotactic factor in the area surrounding the well, granulocyte suspension in medium containing chemotactic factor was placed in an agarose plate which did not contain chemotactic factor. As shown in Table I, granulocyte mobility under a negative concentration gradient of chemotactic factor increased significantly compared to mobility without factor.

Next we examined the influence of a positive concentration gradient toward

TABLE I. GRANULOCYTE MOBILITY UNDER CONDITIONS OF CHEMOTACTIC STIMULATION

Gradient	Distance
No factor (random mobility)	22.9 $\pm$ 6
Negative gradient	33.0 $\pm$ 7
Uniform gradient (10% chemokinesis)	54.5 $\pm$ 5
Positive gradient (chemotaxis)	69.4 $\pm$ 1

<sup>a</sup> Expressed as  $\times 40$  mm.

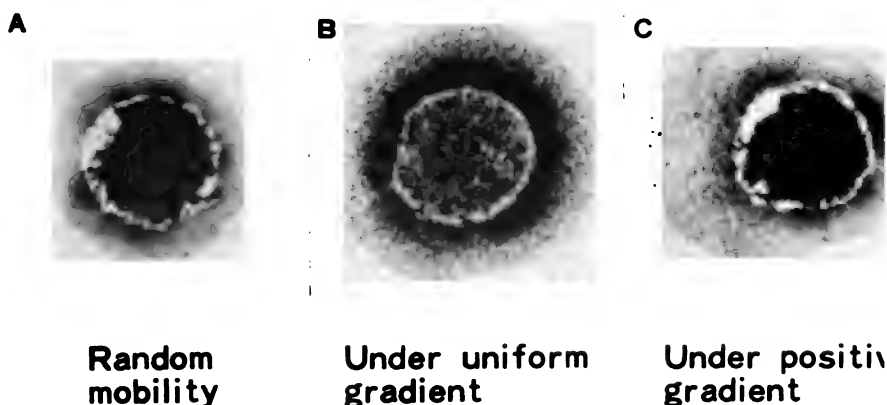


FIG. 2. Three types of granulocyte mobility after three hour culture. Cells were stained by Giemsa solution. A: random mobility, B: chemokinesis, C: chemotaxis, chemotactic gradient was made at the left side.

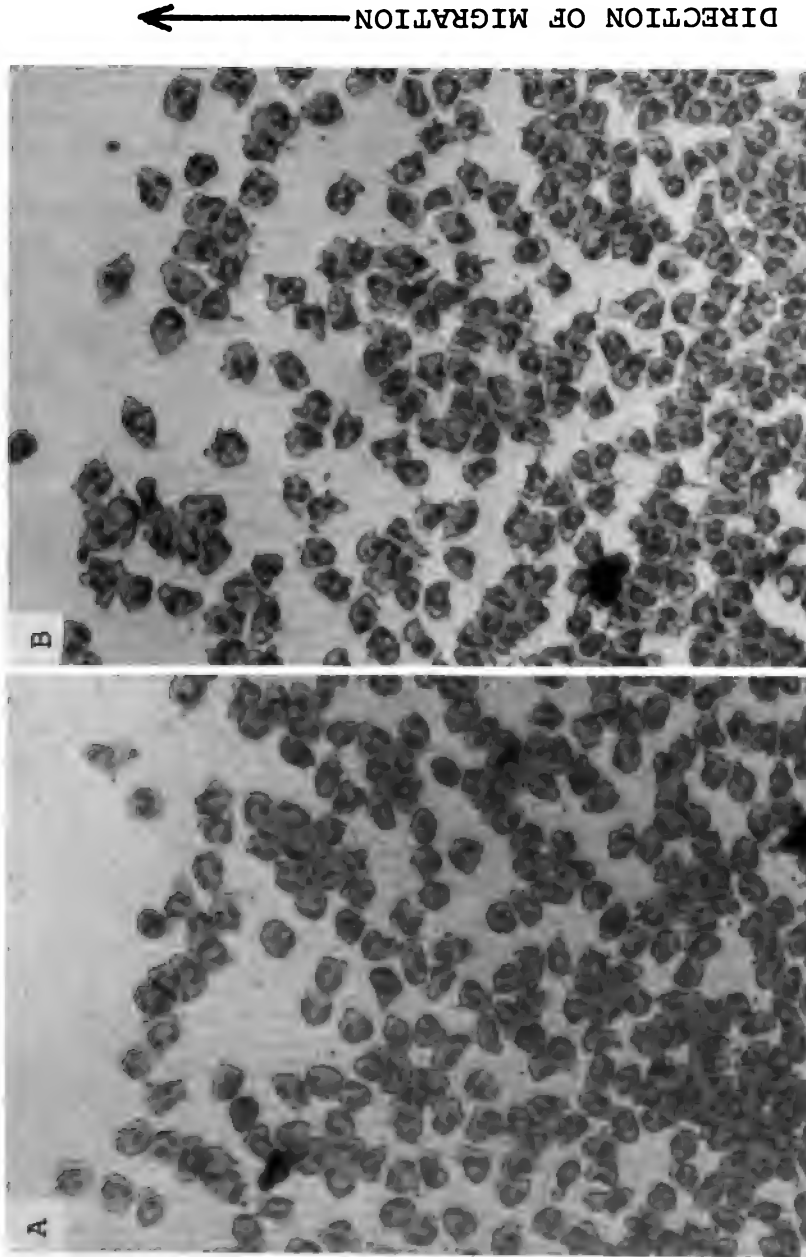


FIG. 3. Morphologies of random mobility (A) and chemokinesis (B). Chemokinesis was measured under 10% chemotactic factor. Cells were stained by Giemsa solution. Cells mobilizing under the influence of chemotactic factor tend to be more irregular in outline than cells mobilizing without the influence of factor.  $\times 400$ .

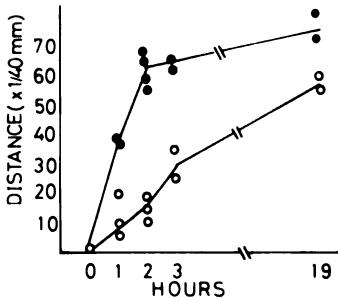


FIG. 4. The time-course of leucocyte mobility. Cells under the influence of 10% chemotactic factor (●) moved rapidly, whereas cells not under the influence of the factor (○) showed a gradual and linear increase.

well in which cell suspensions were placed. Cells exposed to a chemotactic gradient appear to extend further than those under any of the uniform concentrations tested (Table I and Fig. 5). This fact suggests that chemotaxis is also occurring in this condition.

**Effect of preincubation with chemotactic factor on granulocyte mobility.** Table II shows the effect of preincubation of granulocytes with Medium-199 containing 0, 2.5, or 10% of chemotactic factor at 37° for 1 hr, followed by washing with Hanks solution, and resuspension in Medium-199 containing heat inactivated 10% of fetal calf serum. The cells were then placed into the wells in the agarose plate with or without chemotactic factor. Preincubation with chemotactic factor of three concentrations had no influence on chemokinesis observed in agarose containing 5% of chemotactic factor. Furthermore granulocytes preincubated with 10% of chemotactic factor did not show enhanced mobility, namely chemokinesis, when the granulocytes were placed into the wells in agarose not containing chemotactic factor.

**Discussion.** Random mobility and chemokinesis could be observed separately by the agarose plate method. Granulocytes under a

uniform concentration of *E. coli*-derived chemotactic factor moved at a significantly higher rate than in the absence of factor. Morphologically differences were also apparent. Cells showing chemokinesis tend to be irregular in outline, whereas those showing random mobility tend to be rounded.

Nelson *et al.* and Cutler reported that the distance the cells moved toward the outer well in which chemotactic factor was placed, was determined by chemotaxis of granulocytes (6-8). But from the results obtained in our experiments, we conclude that this distance may be based partially on chemokinesis.

The time course of cell mobility triggered by chemokinesis is analogous to that in response to chemotaxis as reported by Nelson *et al.* and Cutler (6, 7). Thus the distance of cells showing chemokinesis as well as those showing chemotaxis increases with the passage of time. In our experiments, there was a dose response relationship between chemo-

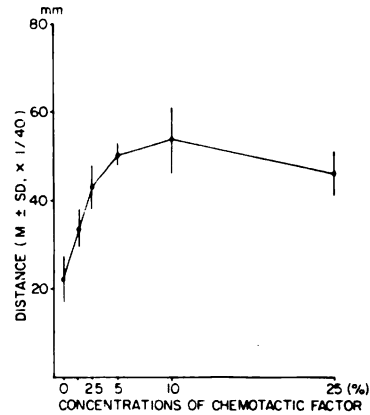


FIG. 5. Relationship between the concentrations of chemotactic factor and the degree of chemokinesis. Leucocyte mobility increased linearly in proportion to the concentrations of chemotactic factor under less than 2.5% of the factor.

TABLE II. EFFECT OF PREINCUBATION WITH CHEMOTACTIC FACTOR ON GRANULOCYTE CHEMOKINESIS.<sup>a</sup>

	Concentration of chemotactic factor with which cells were preincubated		
	0%	2.5%	10%
Mobility in agarose containing 5% factor (M ± SD)	50.9 ± 2.2 n = 5	51.3 ± 3.8 n = 3	53.4 ± 3.3 n = 3
Mobility in agarose containing no factor (M ± SD)	22.9 ± 6.3 n = 9		22.3 ± 11.7 n = 3

<sup>a</sup> Expressed as × 40 mm.

kinesis and the concentration of chemotactic factor, and the degree of chemokinesis was determined by the final concentration of chemotactic factor with which granulocytes came in contact. Thus cells coming in contact with higher concentration of chemotactic factor move more rapidly at random, and can increase their chances of coming in contact with chemotactic factor of higher concentration, because of the enhanced mobility. If this does not occur, namely when they move away from the chemotactic factor, the cells remain in the same location and may regain random movement regardless of whether they have been stimulated or not. Thus the number of granulocytes in the area of lower concentration of chemotactic factor decreases, whereas the number in an area of higher concentration increases. This hypothesis may be supported by another finding in the present experiments. Some enhancement of mobility under a negative concentration gradient of chemotactic factor, which can not be explained by the concept of chemotaxis, suggests that chemokinesis occurs also under a chemotactic gradient. As far as *E. coli*-derived chemotactic factor is concerned, besides a real chemotactic response (1-4, 9, 10), a chemokinetic response may also account for the effect of chemotactic gradient on trapping of cells at the site of inflammation.

The phenomenon of "deactivation" which was shown by Ward and co-workers' study concerning chemotaxis (11), could not be observed in chemokinesis induced by *E. coli*-derived chemotactic factor. It is uncertain whether "deactivation" can be observed also in chemokinesis induced by other chemotactic factor such as complement-derived factors. However, if such a phenomenon occurs in chemokinesis induced by some chemotactic (or chemokinetic) factor, the trapping of cells in an area where high concentration of chemotactic factor is present may be performed more effectively.

We believe that chemokinesis in addition to chemotaxis plays an important role in the defense mechanisms *in vivo*. Further investigation is required to better understand the basic mechanisms involved in the chemotactic (or chemokinetic) response of granulocytes.

The authors gratefully acknowledge the helpful advice of Dr. Paul G. Quie.

**Summary.** Human granulocyte mobility under various conditions of chemotactic stimulus was studied using the agarose plate method. Enhanced mobility was observed when granulocytes were incubated in the agarose plate containing chemotactic factor generated from *E. coli*. A dose response type relationship was observed between the degree of enhanced mobility and the concentrations of chemotactic factor in a range of less than 10%. The rate of mobility was rapid up to 3 hr, after which time it was very slow. Preincubation of granulocytes with chemotactic factor of various concentrations did not have any influence on granulocyte mobility assayed after preincubation. The degree of mobility tends to be determined by the final concentration of chemotactic factor coming in contact with granulocytes. Thus granulocytes under a negative concentration gradient also showed an enhanced mobility. On the basis of these findings, we propose the hypothesis that the accumulation of granulocytes at the site of inflammation can be in part explained by chemokinesis, i.e. enhanced random mobility.

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## Hypophysectomy Alters the Diurnal Food Intake Patterns in Rats (40288)

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Several hypothalamic nuclei have been shown to influence rhythmic physiological processes. Destruction of the suprachiasmatic (1), dorsomedial hypothalamic (DMN) (2) and the ventromedial hypothalamic nuclei (VMN) (3) alter the normal diurnal food intake pattern of rats. Ablation of these nuclei also disrupts the natural corticosterone rhythm of rats (4) (5). Recently several papers (4, 6-9) have concerned themselves with the possible relationship of pituitary hormone rhythms and feeding and drinking patterns.

It has been known for some time that hypophysectomy decreases food consumption of rats. More recently Stephan and Zucker (10) reported that rats did not display a normal diurnal food intake following hypophysectomy combined with ovariectomy. They noted that the nocturnal rhythms in eating and drinking were greatly attenuated following hypophysectomy-ovariectomy. However, since they (10) measured the animals' food intake only at the start and end of the light-dark cycle their data do not reveal the effect of hypophysectomy on meal patterns.

The present study investigated the individual meal patterns of hypophysectomized rats in order to determine frequency, duration and distribution of the meals.

**Materials and methods.** Male hypophysectomized and nonoperated Sprague-Dawley rats were purchased from Simonsen Laboratories Inc., Gilroy, CA. The rats were housed individually under a light:dark (L:D) ratio of 12:12 with lights on at 0600 hr. During the experiment the rats were given a purified diet consisting of: 15% vitamin-free casein, 0.3% L-methionine, 1.0% vitamin premix, 5% salt mixture, 49.05% corn starch, 24.55% sucrose, 5% corn oil and 0.1% of a choline chloride solution. This diet was selected because a powdered diet had previously given more reproducible results than a chow-type diet when measured by the automatic food intake

recorder. The feeder was designed so food could be readily obtained from a glass fiber glass cup designed to prevent spillage. An event marker recorded each time 10% of food was removed from the cup (Rogers and Leung (11) for additional information).

To prevent disturbance of the rats, their room was restricted to 1730-1800 hr. The animals were allowed a 7 to 10 day period to adjust to the room, purified diet and food intake monitoring apparatus to recording food intake patterns. At the end of this period, which was approximately 10 days after the animals were hypophysectomized, food intake patterns were recorded continuously for 3 days.

Any period of food intake in which the time between recordings was defined as a meal. Thus, the number of daily meals could be determined. For statistical analysis the animals were considered hypophysectomized if they did not show weight gains (weight at arrival  $108 \pm 1.9$  g and at end of study  $\pm 2.7$  g) and after histological examination showed no pituitary remnants in the turcica. This yielded a population of hypophysectomized and seven control rats.

The data were analyzed using Student's t test, Mann-Whitney U test and Chi-Square test.

**Results.** The controls ate  $97.6 \pm 1.0\%$  of their food during the light phase and  $5.5\%$  of their food during the dark phase ( $P < 0.001$ ) (Fig. 1). When the number of meals consumed per 24 hr by the hypophysectomized (hypox) rats was compared no significant differences were found (controls  $7.9 \pm 0.6$ ; hypox  $7.4 \pm 0.8$ ). However, the light:dark distribution of the meals was significantly different (Light phase: controls  $0.7 \pm 0.2$  vs hypox  $\pm 0.2$ ,  $P < 0.001$ ; Dark phase: controls  $0.5$  vs hypox  $5.5 \pm 0.7$ ,  $P < 0.05$ ). The average daily intake of the controls was higher than that of the hypophysectomized rats (1

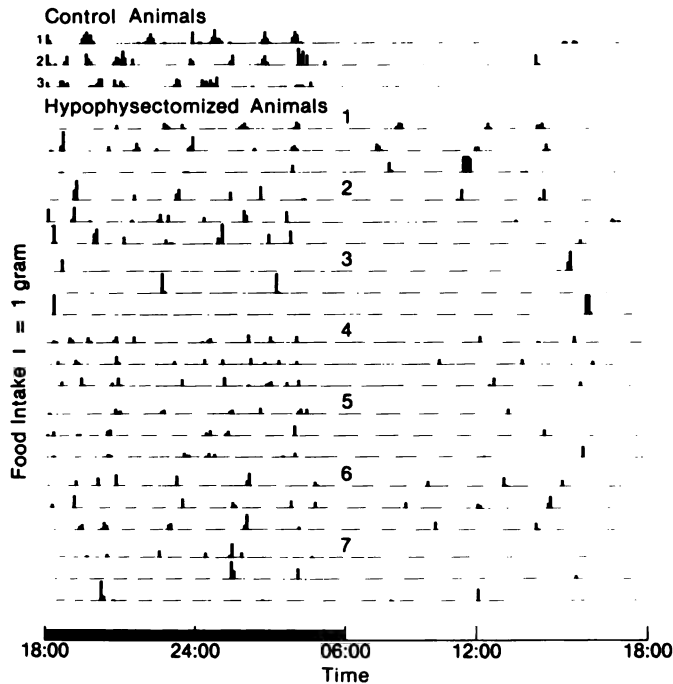


FIG. 1. Twenty four food intake profiles on a representative control animal and seven hypophysectomized rats. The animals' food intake patterns are shown for 3 consecutive days. The dark bar corresponds to the dark period.

0.9 g vs  $6.7 \pm 1.0$  g,  $P < 0.001$ ). The differences in food consumption between the two groups was probably associated with the fact that the controls continued to grow during the adjustment period while growth was arrested in the hypophysectomized animals. The data revealed that both groups ate approximately the same number of meals each day. Thus, the hypophysectomized rats appeared to reduce their daily food intake through a reduction in average meal duration (controls  $15.1 \pm 1.1$  min vs hypox  $4.5 \pm 1.0$  min,  $P < 0.001$ ) and meal size (controls  $2.7 \pm 0.4$  g/meal vs hypox  $1.1 \pm 0.2$  g/meal,  $P < 0.01$ ).

Inspection of the daily feeding patterns (Fig. 1) reveals a pronounced diurnal food intake rhythm in the control animals and an altered pattern in the hypophysectomized animals. While hypophysectomy has statistically altered the animals normal diurnal eating rhythm several of the rats (Fig. 1, rats #1, 2, 5 and 7) still appear to be strongly influenced by the photoperiod. The remaining three hypophysectomized rats showed a much greater altered eating rhythm. The na-

ture of the difference waits to be resolved. A further indication that hypophysectomy has altered the rats normal feeding rhythm is shown in the day to day variation in the percentage of food consumed during the light and dark phases. The day to day variation were computed on each group of animals and then summed as to the period of feeding and analyzed. Control rats showed very little day to day variation in the percentage of food they consumed during the dark and light phase while the hypophysectomized rats showed a great deal of variation (Dark phase: controls,  $\chi^2 = 3.53$ ,  $P > 0.99$  hypox,  $\chi^2 = 100.55$ ,  $P < 0.001$ ).

**Discussion.** Stephan and Zucker (10) showed that a combination of hypophysectomy and ovariectomy altered the normal diurnal food intake pattern of rats. The present study revealed that hypophysectomy alone can modify the normal diurnal feeding pattern of rats.

Hypophysectomy arrests growth and depresses the animals daily food consumption (12). The data presented here reveal that, while the 24-hr meal frequency of the hy-

pophysectomized rats was similar to nonoperated controls, the light-dark distribution of meals was significantly altered by hypophysectomy. Furthermore, total daily food intake was reduced by hypophysectomy because meal duration and size were greatly reduced in the hypophysectomized rats. Thus, hypophysectomized rats appear to decrease their food intake through a reduction in meal size and not meal frequency.

It has been proposed (12) that because hypophysectomy decreases daily food consumption, pituitary hormones might be directly involved in the regulation of feeding behavior. This was challenged (13) on the grounds that hypophysectomy causes a drop in basal metabolic rate and the decrease in food consumption is responding to that decreased basal metabolic need. However, the lower basal metabolism cannot readily account for the fact that the hypophysectomized rats of Stephan and Zucker's study (10) or of the present one, displayed an altered diurnal feeding pattern. One possible explanation is that hypophysectomy removed one or more of the pituitary hormones which help determine the food intake rhythm. In support of the pituitary playing a role in the maintenance of certain consummatory rhythms is the finding that the posterior pituitary hormone, antidiuretic hormone has been shown to be important in maintaining the light-dark distribution of drinking in rats (9).

Rats eat the majority of their food at night, commencing shortly after the start of the dark phase. Increases in several pituitary hormones (6-8, 14) appear to coincide with the onset of normal feeding in rats (10, 15). Corticosterone (14, 15) and prolactin (6, 7) peak prior to onset of the normal feeding period and if rats are fed for only 2 hr per day, the natural rhythms of these hormones are modified (6, 16, 17). Within a short time both hormones show peaks prior to the start of the new feeding period (6, 16, 17) with a reduced peak remaining at the end of the light period. Both the natural corticosterone and prolactin rhythms persist in fasted rats (7). Interestingly, when rats are refed, even after periods of fasting up to 36 hr (18, 19), they consistently consume more when presented with food during the dark phase than when refed during the light phase. Also noteworthy, is

the finding that lesions of the DM VMN disrupt the natural diurnal feeding pattern of mature and weanling rats (2, 3) also altering the normal diurnal corticosterone rhythm (4). In the present study, hypophysectomy, which would necessarily increase prolactin and alter corticosterone levels, seemingly modifies the natural food intake patterns of the animals. However, a study of ours (Bellinger *et al.*, unpublished observations) indicates that adrenalectomy does not alter the diurnal feeding rhythm in rats. This indicates that the corticosterone rhythm is not the cause but only incident to the rats feeding rhythm. Thus some pituitary factor(s) may be responsible for the diurnal maintenance of the diurnal feeding rhythm.

Since on the average the food consumption of the hypophysectomized rats was still influenced by the photoperiod, it appears that some pituitary hormone(s) or some other pituitary factor(s) can only be partially responsible for the maintenance of the normal diurnal feeding rhythm. Finally, it must be considered that hypophysectomy does alter the animal's basal metabolism and this might possibly modify the food intake pattern of the animal.

**Summary.** Hypophysectomy alters the normal diurnal feeding patterns of rats. On the average the hypophysectomized rats consume the greatest percentage of the food during the dark phase. Compared to control rats, hypophysectomized rats eat a similar number of meals each day, however, the amount of food consumed and the duration of the meals are reduced. The pituitary gland appears to be one of the factors involved in sustaining the natural diurnal feeding rhythm.

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# Protein-Calorie Malnutrition Impairs the Anti-Viral Function of Macrophages<sup>1</sup> (40289)

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Human malnutrition is accompanied by decreased resistance to at least certain infectious diseases (1). This enhanced susceptibility may be mediated by such factors as altered complement metabolism (2), deficient cellular immune responses (3, 4) and decreased production of secretory immunoglobulins (5). Little information is available as to whether macrophage antimicrobial mechanisms are also affected by malnutrition. As an important member of primary host defenses and as an effector cell for many of the cellular immune processes, the macrophage plays a critical role in infectious diseases (6). Consequently any degree of impairment of the efficiency with which this cell performs these roles might be expected to result in considerable reduction in host resistance.

Douglas and Schopfer (7) reported that monocyte phagocytic indices are not altered by severe protein-calorie malnutrition. Paswell *et al.* (8), however, noted that phagocytic capacities of macrophages do seem to be impaired in mice which were protein-deprived. Nevertheless, neither of these reports described the total microbicidal capability of the macrophage. Keusch *et al.* have recently reported (9) that macrophages from mice with kwashiorkor kill *Staphylococcus aureus*, *E. coli* and salmonella normally *in vitro*. These authors infer that *in vivo* however, it appears likely that macrophage contributions to host defenses are impaired.

We have recently described a model in mice which demonstrates that age-specific resistance to ip infection with Wesselsbron virus (WBV) is macrophage-mediated (10). Resistance by mice is essentially complete by age 2-3 weeks, and represents the acquired ability of peritoneal macrophages to phagocytose and to destroy infectious virus. The

present report represents studies on the resistance of protein-calorie malnourished mice to WBV infection and whether macrophage antiviral function is concomitantly affected.

**Materials and methods.** *Mice.* Random-bred 3-week old white mice were individually caged and allowed free access to water and food. Experimental mice were placed on protein-depletion diet USP XV composed of 84% white dextrin, 9% corn oil, 4% salt mixture, 2% agar, 1% cod liver oil and vitamin supplement (ICN Nutritional Biochemicals). Control mice were fed a normal protein diet containing 27% casein. Under these conditions normal mice showed a mean increase in body weight of 5.3% after 5 days while protein-depleted mice lost a mean of 12.9% body wt during the same period. Unless otherwise noted resistance to infection was determined in mice that had been fed protein-depletion diet for 5 days and continued on this diet during the observation period.

**Virus.** The source and preparation of WBV was as previously published (10). Infectivity was assayed by inoculating 1-day old mice intracerebrally (i.c.); serial tenfold dilutions were made and each dilution was inoculated into one litter (9-14 mice). End-points were determined by summarizing mortality 14 days later and calculated by the method of Reed and Muench (11).

**Results.** Of 21 normal mice inoculated ip with WBV ( $10^8$  LD<sub>50</sub> as assayed in suckling mice), none developed signs of illness nor died. In contrast, each of 11 protein-depleted mice developed symptoms of encephalitis; three of these mice were sacrificed on day 5 and brain tissue was assayed for WBV while the other 8 mice succumbed within 7 days of inoculation. WBV,  $10^{5.3}$  LD<sub>50</sub> per 0.1 g tissue was recovered from each of the brains tested. One of 12 protein-depleted mice observed as uninoculated controls died after 5 days but no virus could be recovered from its brain.

To study how rapidly susceptibility to WBV developed, a series of mice were inoc-

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lip with WBV at various times before switching them to the protein-depletion diet. The details and results of this experiment are shown in Table I. Mice that inoculated with WBV 3 days or longer before initiating protein depletion showed almost complete resistance to virus. However, susceptibility to ip infection with WBV rapidly decreased in relation to protein depletion such that even animals inoculated one day previously showed decreased resistance to infection. Signs of encephalitis appeared 5–7 days after inoculation in all mice succumbing to infection.

These data implied that some event occurred relatively early in the initial stage of infection that was sensitive to protein depletion. To support this idea it was necessary to determine how soon after ip inoculation virus could be detected in the central nervous system. Groups of mice on normal diet or 5 days on depletion diet were inoculated with  $10^6$  LD<sub>50</sub> of WBV as before, and brain samples from two animals of each group were collected daily thereafter (Table II). In normal mice very small amounts of WBV were recovered 1–3 days postinoculation, while samples collected on days 4–7 contained no detectable virus. Simultaneously collected samples of blood obtained by section of the axil-

lary vessels contained similar concentrations of virus and presumably represented the source of virus present in the brain samples. In contrast, much greater concentrations of WBV could be detected in blood from protein-depleted mice. Brain samples also had concentrations of virus of similar magnitude on days 3 and 4, although the presence of viremia made the origin of this virus uncertain. By day 5, however, the titer of virus in the brain significantly exceeded that in the blood and by day 6 the animals had developed encephalitis.

Unstimulated peritoneal macrophages were collected from normal and from PCM mice and the susceptibility of WBV to inactivation by these cells was studied by the methods previously described (10). Briefly, cells were collected by washing the peritoneal cavity with phosphate-buffered saline (PBS, pH 7.2). The cell suspension was inoculated into glass bottles and allowed to attach for 2 hr at 37°. Nonadherent cells were removed by repeated vigorous washing with PBS. The adherent cells were resuspended by scraping and cultured in medium 199 at a concentration of  $10^6$  cells per ml. WBV was added at a multiplicity of infection of one and allowed to adsorb 1 hr at 37°. The cultures were then washed 3 times and fresh medium 199 was

TABLE I. DEVELOPMENT OF SUSCEPTIBILITY TO WBV IN MICE PLACED ON PROTEIN-DEPLETION DIET BEFORE OR AFTER VIRUS INOCULATION.

	WBV inoculated on day <sup>a</sup>					
	-5	-3	-1	+1	+3	+5
no. mice inoculated	10	10	10	10	10	10
no. mice surviving	10	10	2	0	0	0

<sup>a</sup> Mice were switched from normal to protein-depletion diet on day 0. WBV,  $10^6$  LD<sub>50</sub> (suckling mouse assay) was injected intraperitoneally on days indicated before (minus days) or after (plus days) switching diets. Mortality for group of mice was summarized 14 days after inoculation with WBV.

TABLE II. WBV TITER IN BLOOD AND BRAIN TISSUE AFTER INTRAPERITONEAL INOCULATION OF NORMAL AND PCM MICE.

	Days postinoculation						
	1	2	3	4	5	6	7
Normal: blood <sup>a</sup>	1.2	1.0	1.3	0.6	0	0	0
brain <sup>b</sup>	0.9	1.2	0.3	0	0	0	0
PCM: blood <sup>a</sup>	2.2	3.3	3.3	3.3	4.6		
brain <sup>b</sup>	2.6	2.9	4.1	4.8	8.3		

<sup>a</sup>  $10^6$  LD<sub>50</sub> per 0.05 ml serum.

<sup>b</sup>  $10^6$  LD<sub>50</sub> per 0.1 g tissue.

added at time 0. At intervals some cultures were rapidly frozen and thawed 3 times, debris was removed by centrifugation and the supernatant virus content was assayed. The experiments were run in triplicate and assay results were pooled by summing mortality of the individual titrations. In no instance did the end-points of individual titrations disagree by as much as one  $\log_{10}$  dilution. The results of these experiments are illustrated in Fig. 1. Whereas infectious WBV had completely disappeared by 24 hr in macrophages obtained from normal animals there was an obvious difference in the ability of macrophages from PCM mice to inactivate WBV. This suggests that the extraperitoneal dissemination that occurred to a much greater extent in PCM mice (Table II) resulted from the decreased capacity of local defense mechanisms to inactivate and contain the infectious inoculum. Presumably, macrophages were a major contributor in the population of cells studied.

**Discussion.** The effects of malnutrition on host resistance to virus infections may be variable. Measles infections are a classic example of the increased susceptibility to severe and often fatal effects of disease occurring in the malnourished host (12).

Experimentally, malnourished mice have

been shown to have decreased resistance to coxsackie B3 virus (13) but apparently increased resistance to pseudorabies virus (14). Host resistance to different viruses represents a complex interaction of many mechanisms, each affected to varying degrees by malnutrition. Thus, contrasting results with individual viruses with distinctive pathogenetic schemes is not surprising. The model employed here attempts to isolate the effect of the macrophage.

Although mice normally develop age-specific resistance to WBV the target organ (central nervous system) remains susceptible to infection and disease (10). Thus, the data presented here on the loss of resistance to peripheral (ip) infection in protein-calorie malnourished mice implies that local resistance is impaired, and that moreover the events mediating resistance are rapidly sensitive to the deleterious effects of this malnutrition. In normally nourished mice only limited amounts of virus gain access to the circulation after ip inoculation, and this is apparently below the threshold required to initiate infection in the central nervous system. Local restriction of the virus inoculum was not effective with protein-calorie malnutrition; large amounts of virus appeared in the circulation and encephalitis ensued.

The direct interaction of WBV and macrophages cultured *in vitro* suggested that a primary effect of protein-calorie malnutrition was on the ability of macrophages to restrict WBV infection. Significant levels of infectious virus persisted throughout the time period studied and in fact WBV may have replicated in the macrophages from malnourished mice. This must at least in part account for the susceptibility of these mice to WBV infection. In this regard they would be similar to newborn mice which are susceptible to infection for similar reasons (10). These results suggest that impaired macrophage function is an additional feature of protein-calorie malnutrition that contributes to the susceptibility of such individuals to certain virus diseases. Since the mechanism by which macrophages exert antiviral effects is not understood the cellular basis of the defect is obscure.

**Summary.** Mice which are normally resistant to infection with Wesselsbron (WBV)

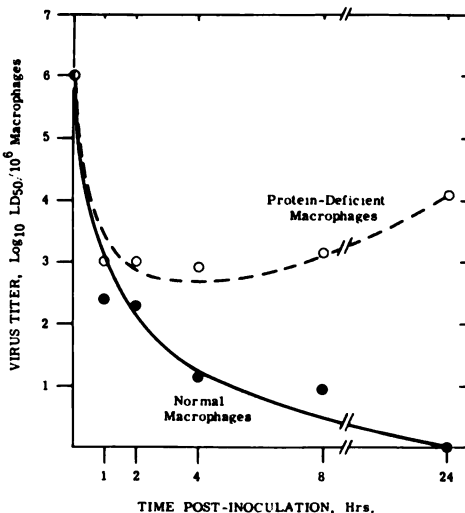


FIG. 1. Infection of macrophages with WBV. Titer of infectious virus present at intervals after inoculating cultures of macrophages obtained from normal and protein-deficient mice.

is became rapidly susceptible to disease to this agent after being placed on protein-depletion diet. After ip inoculation large amounts of virus appeared in the circulation followed by fatal encephalitis. In normally fed mice only small amounts of virus could be detected in blood and no disease developed. This suggested that local defense mechanisms which normally restrict the extent of infection was sensitive to the early effects of protein-calorie malnutrition. That this was at least in part to impaired antiviral action of macrophage under these conditions was confirmed by *in vitro* macrophage studies. Over the course of 24 hr infectious V disappeared after inoculation into cultures of normal macrophages whereas infection persisted at high titers in macrophages from protein-depleted mice.

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# The Effect of Heparin on Growth of Mammalian Cells *in Vitro*<sup>1</sup> (40290)

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Heparin has been used primarily as a therapeutic anticoagulant agent (1), and clinically used to treat inflammatory and allergenic diseases (2). It has also been shown to accelerate recovery of burn patients and promote wound healing in humans and animals (3-5). The mechanism and process of burn and wound healing from these observations is still not well elucidated. It is, therefore, of interest to investigate whether heparin can stimulate proliferation of human skin diploid cells *in vitro* which might be related to the mode of action of the healing process.

The effect of heparin and other acid mucopolysaccharides on the growth of various cell types, mainly malignant cells, has been studied by a number of investigators. The results obtained often have been controversial. Some investigators find inhibitory effects on cell growth (6-8), some stimulatory (8-10), and some report morphological changes (11). Therefore, this study was carried out in an attempt to provide more information about the nature of the effect of heparin on the growth of cultured mammalian cells.

**Materials and methods. Chemicals.** Amino acids, vitamins and newborn calf serum were purchased either from International Scientific Industries, Inc., Cary, IL, or Grand Island Biological Co., Grand Island, NY; prednisolone-21-sodium-succinate (PSS) and *N*-2-hydroxyethylpiperazine - *N'*-2-ethane-sulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; S-210 medium from Grand Island Biological Co.; Waymouth 752/1 dry powder medium from Schwarz/Mann Inc., Orangeburg, NY; fatty acid-free bovine serum albumin (FAF-BSA) from Miles Laboratories, Inc., Elkhart, IN; oleic acid from Nu-Chek Prep, Inc., Elysian, MN; various forms of heparin were kindly supplied by Riker Co., Division of 3M Co., St. Paul, MN;

Calbiochem, La Jolla, CA, Upjohn Co., Kalamazoo, MI; and highly purified heparin was a gift from Dr. J. A. Cifonelli, University of Chicago, Chicago, IL.

**Cell cultures.** The sources of the cells and the methods used for cultivation were the same as described previously. Monkey kidney (MK-2) cells were cultivated as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% newborn calf serum (MEM<sub>5</sub>) (12). Novikoff hepatoma cells were grown in shaker culture in S-210 medium (13). Human prepuce cells were grown as monolayers in Eagle's MEM medium (14) supplemented with 10% newborn calf serum (MEM<sub>10</sub>) and baby hamster kidney cells (BHK-21) were grown in shaker culture using a modified Waymouth 752/1 medium (15).

**Growth of cells in the presence of heparin.** Prepuce cells were grown in Eagle's MEM supplemented with 4% newborn calf serum (MEM<sub>4</sub>) or MEM<sub>10</sub> in the presence of a wide range of heparin. Hanks' balanced salt solution (BSS) was used as a base (16). The cells were used at an initial density of  $2.0-3.0 \times 10^5$  cells/flask in a volume of 4 ml and were placed in 25 cm<sup>2</sup> cell culture flasks (Corning Glass Works, Corning, NY). The cells were incubated at 37° for 6-10 days and were enumerated at varying intervals of time after trypsinization with the aid of a Coulter counter.

Thirty ml of BHK-21 cells were suspended in modified Waymouth 752/1 medium (15) containing 2.5% newborn calf serum and different amounts of heparin. The initial cell population contained  $3.5 \times 10^5$  cells/ml and were incubated at 37° in a New Brunswick gyratory shaker. At 0, 24, 48, 72 and 96 hr the cells were enumerated with a Coulter counter.

An initial population of  $3.0 \times 10^5$  MK-2 cells in 4 ml of modified Waymouth 752/1 medium (15) was added to 25 cm<sup>2</sup> flasks. In order to establish monolayers of cells, the medium was supplemented with 1% newborn

<sup>1</sup> This work was supported in part by the Office of Naval Research, Contract Nos. N00014-75-C-0903, NR202-071, and by The Hormel Foundation.

a. After 24-hr incubation at 37°, the was discarded and the cells rinsed  
b. Fresh modified Waymouth me-  
tout the serum supplement contain-  
ent amounts of heparin were added  
ls. The cells were incubated at 37°  
l were enumerated at varying inter-  
ne.

off hepatoma cells were grown in  
lture in S-210 medium in the pres-  
fferent amounts of heparin. A start-  
y of  $2 \times 10^5$  cells/ml was incubated  
r 4 days. Cell numbers were deter-  
ery 24 hr.

ed effect of heparin and PSS on the  
'BHK-21 and prepuce cells. Varying  
of PSS and heparin were added in  
binations to the growth medium  
te BHK-21 cells in shaker cultures  
uce cells in monolayers. The cell  
as measured at varying intervals of  
: procedures were the same as de-  
bove for testing the effect of heparin

imum of two independent experi-  
re performed for all studies. Each  
: was carried out in triplicate, and  
counts were made on each sample.  
ts were analyzed for significant dif-  
y using a student's t test.

. The effect of Riker's hog mucosal  
on the growth of prepuce cells culti-  
MEM<sub>10</sub> is shown in Table I. There  
immediate differences observed in  
etween control and heparin-treated  
ng the first 3 days after incubation.  
, the cells cultivated in the medium  
g 5 and 10 µg/ml of heparin had an  
of 30% and 23%, respectively, in cell  
over that of the control cells. There  
dest increase in the number of cells

grown in medium containing 15 and 20 µg/ml  
of heparin, whereas the cells treated with 80  
µg/ml of heparin had a 21% decrease in cell  
population when compared to control cells.  
The population of cells treated with 0, 5, 10  
and 20 µg/ml of heparin began to decline  
after day 5, whereas cells treated with 15 and  
80 µg/ml of heparin continued to increase in  
cell number.

When prepuce cells were cultivated in  
MEM<sub>4</sub> after initially incubating the cells in  
MEM<sub>10</sub> for 24 hr, no differences in the growth  
between heparin-treated and untreated cells  
were observed until 8 days after incubation  
(Table II). On day 8, cells treated with 5  
µg/ml of heparin showed a 90% increase in  
cell numbers over that of the untreated cells.  
Cells treated with higher concentrations of  
heparin which were less stimulatory than the  
cells treated with 5 µg/ml of heparin showed  
an increase of about 35% in population. On  
day 10, the cells treated with 5, 10 and 15  
µg/ml of heparin all showed about a 30%  
increase in cell number over the untreated  
cells. Cells treated with 80 µg/ml of heparin  
had about the same growth rate as that of the  
untreated cells.

Various heparins with different anticoag-  
ulant activity obtained from Upjohn Co. and  
Wilson Labs and further purified by J. A.  
Cifonelli showed similar stimulatory effects  
on the growth of prepuce cells (Table III).  
Each of the three heparins at a concentration  
of 5 µg/ml increased the number of cells  
about 30–50% from day 5 to 8 after incuba-  
tion.

Heparins from different sources at a con-  
centration of 5 µg/ml showed similar stimu-  
latory effect on the growth of prepuce cells  
(Table IV), except there was slightly higher  
cell population when the cells were grown in

I. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM SUPPLEMENTED WITH 10% NEWBORN CALF SERUM.

Heparin (µg/ml)					
0	5	10	15	20	80
1.33 ± 0.03 <sup>a</sup>	1.27 ± 0.03	1.30 ± 0.00	1.27 ± 0.07	1.17 ± 0.03	1.30 ± 0.12
2.97 ± 0.09	3.40 ± 0.21	3.20 ± 0.10	2.60 ± 0.17	2.70 ± 0.27	2.63 ± 0.18
4.70 ± 0.10	6.10 ± 0.35 <sup>b</sup>	5.77 ± 0.22 <sup>b</sup>	5.03 ± 0.07	5.30 ± 0.20	3.73 ± 0.70
4.20 ± 0.29	5.67 ± 0.35 <sup>b</sup>	5.30 ± 0.47	6.20 ± 0.31 <sup>b</sup>	4.53 ± 0.29	4.83 ± 0.46

e cell number  $\times 10^5/\text{flask}$  (25 cm<sup>2</sup>)  $\pm$  SEM from three flasks each counted in triplicate.

antly different from control ( $P < 0.05$ ). These data are typical results from a minimum of three  
t experiments.

TABLE II. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM.

Day	Heparin ( $\mu\text{g/ml}$ )					
	0	5	10	15	20	80
1	1.50 $\pm$ 0.07 <sup>a</sup>					
2	2.00 $\pm$ 0.06	1.93 $\pm$ 0.09	1.67 $\pm$ 0.07	1.93 $\pm$ 0.15	1.93 $\pm$ 0.07	2.23 $\pm$ 0.07
4	2.77 $\pm$ 0.03	2.90 $\pm$ 0.10	2.73 $\pm$ 0.07	2.73 $\pm$ 0.22	2.53 $\pm$ 0.07	2.37 $\pm$ 0.03
6	2.83 $\pm$ 0.03	3.17 $\pm$ 0.35	3.17 $\pm$ 0.19	3.03 $\pm$ 0.18	3.37 $\pm$ 0.34	2.60 $\pm$ 0.06
8	2.83 $\pm$ 0.03	5.40 $\pm$ 0.32 <sup>b</sup>	3.60 $\pm$ 0.36	4.10 $\pm$ 0.72	3.83 $\pm$ 0.52	3.27 $\pm$ 0.43
10	4.87 $\pm$ 0.09	6.43 $\pm$ 0.23 <sup>c</sup>	6.40 $\pm$ 0.46 <sup>c</sup>	6.30 $\pm$ 0.15 <sup>b</sup>	5.77 $\pm$ 0.09 <sup>b</sup>	4.83 $\pm$ 0.19

<sup>a</sup> Average cell number  $\times 10^5/\text{flask}$  ( $25\text{ cm}^2$ )  $\pm$  SEM from three flasks each counted in triplicate.

<sup>b</sup> Significantly different from control ( $P < 0.01$ ).

<sup>c</sup> Significantly different from control ( $P < 0.05$ ). These data are typical of results from three independent experiments.

TABLE III. EFFECT OF HEPARINS (5  $\mu\text{g/ml}$ ) WITH DIFFERENT SPECIFIC ACTIVITIES ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM.

Day	Heparins			
	Control	A <sup>a</sup>	B <sup>b</sup>	C <sup>c</sup>
1	1.37 $\pm$ 0.09 <sup>d</sup>			
3	3.07 $\pm$ 0.15	3.27 $\pm$ 0.20	3.17 $\pm$ 0.13	3.47 $\pm$ 0.29
5	3.17 $\pm$ 0.09	4.57 $\pm$ 0.23 <sup>c</sup>	4.33 $\pm$ 0.19 <sup>c</sup>	4.27 $\pm$ 0.22 <sup>c</sup>
6	4.03 $\pm$ 0.12	6.00 $\pm$ 0.40 <sup>c</sup>	5.37 $\pm$ 0.02 <sup>c</sup>	5.63 $\pm$ 0.30 <sup>c</sup>
8	5.20 $\pm$ 0.15	7.10 $\pm$ 0.32 <sup>c</sup>	6.80 $\pm$ 0.46 <sup>c</sup>	6.63 $\pm$ 0.09 <sup>c</sup>

<sup>a</sup> A: Beef lung heparin from Upjohn Co. further purified by gel filtration on Sephadex G-75 by J. A. Cifonelli (specific activity of 144  $\mu\text{g/mg}$ ).

<sup>b</sup> B: Beef lung heparin from Wilson Labs further purified by J. A. Cifonelli (specific activity of 180  $\mu\text{g/mg}$ ).

<sup>c</sup> C: Beef lung heparin from Wilson Labs (specific activity of 110  $\mu\text{g/mg}$ ).

<sup>d</sup> Average cell number  $\times 10^5/\text{flask}$  ( $25\text{ cm}^2$ )  $\pm$  SEM from three flasks each counted in triplicate.

<sup>e</sup> Significantly different from control ( $P < 0.05$ ).

<sup>f</sup> Significantly different from control ( $P < 0.01$ ). These data are typical from three independent experiments.

TABLE IV. EFFECT OF VARIOUS HEPARINS (5  $\mu\text{g/ml}$ ) ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 10% NEWBORN CALF SERUM.

Day <sup>a</sup>	Heparins				
	A	B	C	D	E
1	1.17 $\pm$ 0.03 <sup>b</sup>	1.17 $\pm$ 0.03	1.27 $\pm$ 0.03	1.10 $\pm$ 0.06	1.23 $\pm$ 0.03
3	1.90 $\pm$ 0.06	2.30 $\pm$ 0.10 <sup>c</sup>	2.23 $\pm$ 0.09	2.57 $\pm$ 0.20 <sup>c</sup>	2.90 $\pm$ 0.10 <sup>c</sup>
5	2.67 $\pm$ 0.13	3.13 $\pm$ 0.24	3.27 $\pm$ 0.35	3.00 $\pm$ 0.06	3.77 $\pm$ 0.22 <sup>c</sup>
7	3.17 $\pm$ 0.18	3.73 $\pm$ 0.19	5.13 $\pm$ 0.39 <sup>c</sup>	4.23 $\pm$ 0.23 <sup>c</sup>	4.10 $\pm$ 0.25 <sup>c</sup>
9	3.23 $\pm$ 0.09	4.10 $\pm$ 0.17	4.07 $\pm$ 0.03 <sup>d</sup>	4.03 $\pm$ 0.28	4.40 $\pm$ 0.06 <sup>d</sup>

<sup>a</sup> A: Control, B: Riker's hog mucosal heparin, C: Riker's beef lung heparin, D: Riker's crude hog mucosal heparin, E: Calbiochem's hog mucosa heparin.

<sup>b</sup> Average cell number  $\times 10^5/\text{flask}$   $\pm$  SEM from three flasks each counted in triplicate.

<sup>c</sup> Significantly different from control ( $P < 0.05$ ).

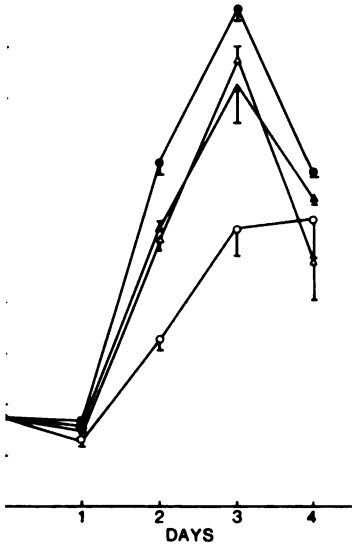
<sup>d</sup> Significantly different from control ( $P < 0.01$ ). These data are typical from three independent experiments.

the medium containing heparin from porcine intestinal mucosa from Calbiochem.

Heparin showed little or no effect on the growth of MK-2, BHK-21 and Novikoff rat hepatoma cells, when the cells were cultivated in the medium containing several concentrations of newborn calf serum in the presence

of a wide range of concentrations of different heparins.

PSS at concentrations of 0.5  $\mu\text{g/ml}$  and 2  $\mu\text{g/ml}$  inhibited the growth of BHK-21 (50%) and prepuce (25%) cells, respectively (Figs. 1 and 2). In an attempt to reverse this inhibitory effect of PSS on cell growth, hog



Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of BHK-21 cells in shaker cultures. 30 ml of a cell suspension of Waymouth 752/1 medium containing an inoculation of  $3.5 \times 10^6$  cells/ml was placed in 125 ml screw-cap flasks. PSS alone or a combination of PSS and heparin was added to the medium. Cells were incubated at  $37^\circ$  and enumerated with a Coulter counter at varying intervals of time.  $\bullet$ — $\bullet$ , 0  $\mu$ g/ml PSS;  $\circ$ — $\circ$ , 0.5  $\mu$ g/ml PSS;  $\blacktriangle$ — $\blacktriangle$ , 0.5  $\mu$ g/ml PSS + 5  $\mu$ g/ml heparin;  $\triangle$ — $\triangle$ , 0.5  $\mu$ g/ml PSS + 0.5  $\mu$ g/ml heparin. Vertical bars represent standard error of the mean.

heparin was added into the culture. It was found that cells grown in the medium containing both PSS and heparin at the same cell numbers as those in the medium containing no PSS (Figs. 1 and 2), reversal of heparin on the inhibitory PSS.

**Discussion.** Previous results about the effect of heparin on cell growth in and other acid mucopolysaccharide cell growth have been equivocal. (17) first claimed inhibition of mitosis of art fibroblasts and concentrations of heparin of varying from 20–500  $\mu$ g/ml. (Cos-  
(18) reported cytotoxic action of heparin of 100  $\mu$ g/ml using Syrian hamster sarcomas. Lippman (8) demonstrated that at 50  $\mu$ g/ml inhibited growth of "L" cells. King *et al.* (18) found that at 1–1000  $\mu$ g/ml showed little effect on division of mouse "L" cells grown in shaker cultures.

Our data showed that heparin appears to promote the growth of prepuce cells but did not stimulate growth of BHK-21, MK-2 or Novikoff rat hepatoma cells. Takeuchi (10) noted that acid mucopolysaccharides have some promoting activity on tumor growth. Ozzello *et al.* (19) reported the growth promoting activity of acid mucopolysaccharides on a strain of human mammary carcinoma cells. They ascribed this action to the negative electric charge and the viscosity of acid mucopolysaccharides.

The controversy about the action of heparin on the cell growth is presumably due in part to different dosages of heparin and the cell types used. Heparin in high concentration can be inhibitory to the growth of cells cultivated *in vitro*. If the amount of heparin is maintained at a dose that just inhibits coagulation (2  $\mu$ g/ml), it seems to be relatively noncytotoxic (11), and perhaps even stimulatory to cell growth. Zakreowski (20) claimed that the Jensen sarcoma in tissue culture was inhibited by heparin, but empha-

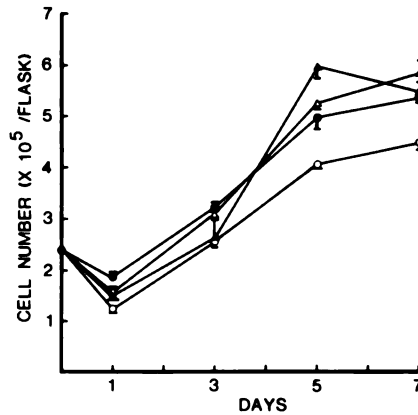


FIG. 2. Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of prepuce cells cultivated in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM<sub>10</sub>). Prepuce cells at an initial density of  $2.3 \times 10^5$  cells/flask in 4 ml of MEM<sub>10</sub> medium were placed in 25 cm<sup>2</sup> cell culture flasks. PSS alone or a combination of PSS and heparin was added to the medium. The cells were incubated at  $37^\circ$  for 7 days and were enumerated with a Coulter counter at varying intervals of time after trypsinization.  $\bullet$ — $\bullet$ , 0  $\mu$ g/ml PSS;  $\circ$ — $\circ$ , 2  $\mu$ g/ml PSS;  $\blacktriangle$ — $\blacktriangle$ , 2  $\mu$ g/ml PSS + 5  $\mu$ g/ml heparin;  $\triangle$ — $\triangle$ , 2  $\mu$ g/ml PSS + 20  $\mu$ g/ml heparin. Vertical bars represent standard error of the mean.

sized that this drug was much less effective on normal embryonic tissue.

Medium supplemented with low amounts of serum (4%) was used in one set of experiments to hopefully show a growth stimulatory effect when supplemented with heparin. Growth of prepuce cells might then be magnified when cells were subliminally starved (21). From the results shown in Table II, about 90% increase of cells was observed compared to the control in the presence of 5  $\mu$ g heparin/ml at day 8. Medium containing 2% serum was also tested. No stimulatory effect of heparin was noted when prepuce cells were grown in this medium. Takeuchi hypothesized (10) that acid mucopolysaccharides did not serve as a nutritional component for cell growth but protects the cell surface and promotes the exchange of various metabolites. Our observation indirectly further supports this hypothesis.

Cell populations from monolayer cultures were found to drop in the first 24-hr incubation. This probably is attributed to cell lysis during the trypsinization process. Therefore, the baseline data for all experiments was best interpreted after 24 hr cultivation.

It has been reported that heparin in animal experiments could interact with steroid hormones(2). Our data demonstrated that heparin reversed the inhibitory effect of PSS on the growth of prepuce and BHK-21 cells cultivated *in vitro*. This test system could be used to indirectly show heparin effects on cell growth when little or no activity was noted by heparin directly. This observation confirms the hypothesis of Dougherty and Dolonitz (2).

A question had been raised whether trace metal contaminants or other unknown contaminants of heparin might be responsible for its activity in aiding burn repair. When crude, commercial grade and highly purified heparins from hog mucosa and/or beef lung sources were tested for promoting cell growth, no differences were found in the activity, which seemed to negate the role of heparin contaminants in the cell culture detection systems used.

Since heparin and heparin-like components are normal constituents of the blood and cells of higher animals, it is not surprising to find that heparin at a physiological level is

harmless and even stimulatory to the growth *in vitro*.

**Summary.** The effect of heparin on the growth of four cell types cultivated *in vitro* has been investigated. The results suggest heparin appears to have some growth promoting effects on prepuce cells, while it showed little effect on the growth of No. 1 hepatoma, monkey kidney and baby hamster kidney cells. Heparin reversed the inhibitory effect of prednisolone-21-sodium-succinate on the growth of prepuce and baby hamster kidney cells.

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# Immune Interferon Activates Cells More Slowly Than Does Virus-Induced Interferon (40291)

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Three antigenically different types of interferon have been found: (a) a 27000-30000 MW protein produced by somatic cells (fibroblast interferon) stimulated by viruses, (b) an interferon produced by leukocytes (leukocyte interferon) also stimulated by viruses (VIF), and (c) immune interferon (IIF), produced by lymphocytes following activation by mitogens or by specific antigenic stimulation (1-3). While the biochemical and biological properties of VIF have been extensively explored and many aspects of its mechanisms of production and action have been clarified, the properties of IIF, especially the mechanisms of activation of the cells, are as yet poorly understood.

Several differences in function between IIF and VIF have been noted. It has been reported that: preparations of IIF exert higher antitumor and immunoregulatory activity as compared with VIF (4, 5), VIF immunosuppressive action but not mitogen induced IIF action is blocked by mercaptoethanol (6), and IIF showed, at least in one system, a decreased ability to inhibit virus yield relative to VIF (7). It seems then reasonable that, since the different types of IF have different mechanisms of induction and show differences of biologic activity, they are likely to manifest important differences in molecular and cellular reactivity (8-10). Since information on this subject could lead to a better understanding of the mechanism of action (antiviral, antitumor, immunoregulatory) of the different types of IF, we have undertaken a comparative study on cellular activation by VIF and IIF.

In previous studies (11, 12) we showed that a very brief reaction (minutes) between VIF and cells at 37° rapidly results in cellular activation which, after removal of IF, is fol-

lowed 30 min later by the transcription translation of mRNA for the antiviral protein responsible for the cellular antiviral state. The present study is a comparison of these kinds of cellular activation using IIF and VIF.

**Materials and methods.** Human leukocyte interferon ( $10^6$  units/mg protein), induced Sendai virus, was obtained from the Anti-Substances Program, NIAID, NIH, and produced by methods previously described (13). Mouse fibroblast interferon was obtained from the mouse C243 cell line induced with Newcastle Disease Virus as previously described ( $10^3$  units/mg protein; 14). Human immune interferon ( $10^{2.4}$  units/mg protein) was obtained from normal lymphocyte cultures stimulated for 4 days with staphylococcal enterotoxin A (SEA). Mouse immune interferon ( $10^2$  units/mg protein) was obtained from mouse splenic cell cultures stimulated with SEA (15). Virus-type interferons were shown to be resistant to 5 days exposure at pH 2 and completely neutralized by specific antibody. Immune interferons were instable at pH 2 and not significantly neutralized by antibody to virus-type interferon (15). Interferon and interferon-induced antiviral activity were measured by the inhibition of yield of Sindbis virus (human interferon) or GD7 virus (mouse interferon) hemagglutination in a single cycle yield assay (16) employing tube cultures of human diploid foreskin cells, HF 19, or mouse L cells, strain CCL-1. Interferon titers are expressed as human or mouse reference units. Temperature control at 37° for short periods of time was effected in a waterbath and longer incubations were effected in a 37° incubator containing 4% CO<sub>2</sub> as previously described (11).

**Results. Development of antiviral resistance in cells treated with virus-induced or immune interferon.** The results of a representative experiment carried out with human leukocyte and immune IFs are shown in Fig. 1. I

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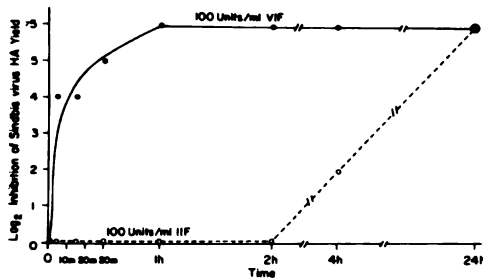


FIG. 1. Development of antiviral activity in human diploid foreskin cells treated for various periods of time with 100 units of either virus-induced or mitogen-induced human interferon.

types of IF were applied at a concentration of 100 reference units per ml as previously described (11). At preestablished times the interferon was removed and the cultures were washed 4 times and challenged with virus (multiplicity of infection, 10). After 1 hr for viral adsorption the cultures were washed 3 times and incubated for 18 hr before titration of viral yield. A control titration of the level of IF activity was included in every experiment. It may be seen that the cell cultures treated with VIF developed substantial resistance after a 5-min treatment and that the degree of resistance to Sindbis virus replication continued to rise thereafter so that 1 hour later it was greater than the measurable level. However, IIF did not induce detectable resistance over 2 hr, and marginal resistance was produced only after 4 hr. The expected degree of antiviral activity was induced after 24 hr treatment.

Similar results were obtained for mouse L cells treated with 300 reference units of either virus induced or immune mouse IF and challenged with GD-7 (Fig. 2). Additionally the same type of kinetics was observed for two more virus-cell systems: vesicular stomatitis virus (human cells, multiplicity of infection, 10) and mengovirus (L cells, multiplicity of infection, 0.1).

Since IIF preparations had a much lower specific activity as compared with VIF preparations, the possibility that some contaminant present in IIF could affect the rate of cellular activation was examined. Specifically cell cultures were treated either with 100 units of VIF, 100 units of IIF or 100 units of VIF plus 100 units of IIF. The cultures were then challenged after 15 min, and 4 hr to deter-

mine whether protection in the cultures treated with both types of IF developed according to the kinetics of development of VIF or IIF. The results (Table I) showed that the rapid kinetics of development of the antiviral state induced by VIF was not slowed by the presence of IIF. The same results were obtained either when the two types of interferon were mixed before addition to the cells or when either type was added immediately before the other.

**Binding of interferon to cells at 37.** It has been shown that cells treated with VIF bind interferon molecules very rapidly (17-21). However data on cellular binding of IIF are not yet available. Since the lack of rapid cellular activation by IIF, as compared with VIF, could be due to different kinetics of cellular binding, experiments were designed to establish the extent of binding of two types of IF under the conditions of rapid activation.

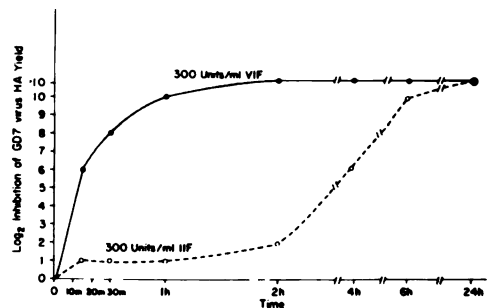


FIG. 2. Development of antiviral activity in mouse L cells treated for various periods of time with 300 units of either virus induced or mitogen induced mouse interferon.

TABLE I. INDUCTION OF THE ANTIVIRAL STATE BY VIRUS-INDUCED INTERFERON, IMMUNE INTERFERON, OR A COMBINATION OF BOTH.

Species of interferon	Type of interferon	Inhibition of virus yield* after treatment for	
		15 min	4 hr
Human	VIF	1.9	2.1
	IIF	0.0	0.8
	VIF + IIF	1.9	2.3
Mouse	VIF	4	>7
	IIF	<1	2
	VIF + IIF	4	>7

\*  $\text{Log}_{10}$  inhibition of Sindbis virus PFU yield (human interferons) or  $\text{log}_2$  inhibition of GD-7 virus HA yield (mouse interferons).



Specifically, 0.5 ml of medium containing 1000 reference units per ml of either virus-induced or immune mouse IF were applied to duplicate tube cultures of L cells maintained at 37°. After different periods of time, one group of cultures was washed 6 times with Earle's balanced solution, refed with 0.5 ml of Eagle's medium, and frozen-thawed 3 times to release cell-associated IF. The fluids were then assayed for IF. The results of a representative experiment are shown in Table II.

It may be seen that binding of both VIF and IIF was essentially maximal after 5 minutes incubation at 37° and that the amount of bound IF remained unchanged thereafter. There was no significant difference between the amount of VIF and IIF bound at each time. The IF associated with the cells was approximately 0.6–1.2% of the total IF applied to the cultures. This finding substantially agrees with previous observations on cellular binding of VIF (16–21) and shows that binding of IIF occurs at a similar rate and to a similar degree.

*Discussion.* It has been previously shown that the development of the antiviral state in cells treated with VIF is triggered immediately by a very brief interaction between IF and cells and continues when the IF is removed from contact with the cells by washing and antibody inactivation (22). This finding has been confirmed for VIF by the data presented in this paper. However IIF, assayed under identical experimental conditions, failed to immediately activate cells after brief contact. Thus in both the human and mouse systems, detectable antiviral resistance was induced by IIF only after several hours of incubation at 37°. The different kinetics of cellular activation by the two types of IF may be due to: (a) Difference of availability of cellular receptors, (b) the presence in the IIF preparation, and not in the VIF preparations, of substances capable of retarding expression of interferon activity under the present experimental conditions, and (c) a different mechanism of activation of the antiviral state.

Studies of cellular binding of the two types of IF did not show any significant difference between their binding activity, suggesting that differences of association by the two IFs with the cell may not play an important role

TABLE II. CELLULAR BINDING OF VIRUS-INDUCED IMMUNE MOUSE INTERFERON.

Type of Interferon	Units of interferon associated with cells after (min)			
	5	10	15	30
Virus-induced	6	12	10	12
Immune	8	10	10	12

in establishing the different kinetics of activation. However, it should be borne in mind that this relatively durable binding which is usually measured (17–21) may not reflect a transient cell-activating event by which induces rapid resistance (22). Specifically has been shown previously that firm binding to the cell surface is not required for the induction of the antiviral state by VIF. The present finding of different kinetics of activation despite equal kinetics of binding further supports that conclusion.

The hypothesis that a component of IIF preparation could interfere with the action of the VIF molecule seems less likely since the presence of the slow acting component in preparation did not inhibit the rapid action by VIF. However this experiment does not eliminate the possibility of the presence of a substance which only inhibits the action of IIF. Further studies with purified preparations of IIF could test this possibility.

The hypothesis that VIF and IIF may induce the antiviral state through different mechanisms appears likely and deserves further study. If the same biological activity be evoked through different activation processes, the finding may provide a useful working model for studying several critical activities, such as cellular regulation of expression, regulation of gene products, cell membrane receptor functions. Additionally, the different kinetics of activation of the antiviral state by the two types of IF provide a simple and rapid method to differentiate them.

*Summary.* The kinetics of activation of the antiviral state by virus induced interferon and by mitogen-induced immune interferon have been studied comparatively. It has been found that both human and murine virus induced interferons are able to activate the antiviral state after a brief (minutes) contact with the cells. In contrast, several hours

quired for both human and mouse immune interferons to induce a comparable level of antiviral resistance. Experiments measuring the binding of the two interferons to cells showed that there was no significant difference in the rate and degree of binding, suggesting that a different total association of interferon with cells could not account for the slower kinetics of activation by immune interferon. Additionally, the possibility that some contaminants present in the immune interferon preparation could nonspecifically interfere with the rapid induction phenomenon is not supported by the finding that the rapid kinetics of cell activation by virus-induced interferon was not modified by the presence of immune interferon. The interesting possibility which remains is that the two interferons may activate cells by different mechanisms.

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## Competition Binding Assay Using *o*-Methyl-[<sup>3</sup>H]-Demethyl- $\gamma$ -Amanitin for Study of RNA Polymerase B (40292)

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The understanding of RNA synthesis and processing, and the enzymes and control mechanisms involved is of central importance in biology. The simplest and most reliable criterion for classification of eukaryotic RNA polymerases is their sensitivity to the fungal toxin  $\alpha$ -amanitin.  $\alpha$ -Amanitin and other naturally occurring amatoxins, as well as their synthetic derivatives, are of particular interest as molecular probes in the study of transcription. They bind very tightly to the polymerase molecule at a site separate from that which binds to the template DNA and product RNA (1, 2). This interaction does not affect the stability of the transcription complex formed between the enzyme and template nor does it interfere with the binding of precursor nucleotide triphosphates (1, 2).

A radioactive derivative of  $\alpha$ -amanitin was synthesized by Wieland and Fahrmeir for use in their structural studies of the molecule (3). The method of synthesis as reported used large amounts of the parent compound and employed various destructive analytic techniques to study placement of functional groups in non-radioactive intermediate compounds and in the labeled end product. To permit the synthesis of a radioactive derivative of  $\alpha$ -amanitin from a small amount of commercially available starting material their procedure was modified, and several new methods for analyzing the unlabeled intermediate compounds and the end product were introduced. This should enable more biologists to avail themselves of this powerful tool.

To demonstrate radiochemical purity and ensure reactivity of the labeled end product, a competition assay was developed. The assay demonstrates that the labeled derivative, *o*-methyl-[<sup>3</sup>H]-demethyl- $\gamma$ -amanitin, binds to the same site as  $\alpha$ -amanitin when reacted with either purified or crude preparations of RNA-polymerase B. Since this technique es-

tablishes that the labeled and unlabeled compounds are essentially interchangeable, it allows the study of amatoxin binding over wider ranges of ligand concentration than heretofore possible when radioactive material was used alone.

**Methods and materials.** To synthesize the first intermediate, *o*-methyl- $\alpha$ -amanitin, 5 mg of  $\alpha$ -amanitin were dissolved in 4 ml of anhydrous methyl alcohol. This was added to 3 ml of an etheric solution of diazomethane generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the outer vessel of an MNNG diazomethane generator. The vessel was stoppered immediately and the reaction mixture was kept at room temperature for 2 hr. The solvents were then evaporated *in vacuo*, the remaining residue was resuspended in a small volume of methanol/H<sub>2</sub>O (1:1) and chromatographed on a column of Sephadex LH-20 (1.8  $\times$  100 cm) with methanol/H<sub>2</sub>O (1:1) (3). Column effluents were monitored at 310 nm with an Isco UA-5 uv monitor. Fractions with uv absorbing material were collected, solvents evaporated *in vacuo*, and the remaining residues redissolved in 1 mg H<sub>2</sub>O. Concentration of the products was determined spectrophotometrically (1, 3, 4).

The second non-radioactive intermediate, *o*-methyl-aldoamanitin, was synthesized via periodate oxidation of *o*-methyl- $\alpha$ -amanitin (3). This was accomplished by the addition of 0.48 mg sodium periodate dissolved in 1 ml water to 2.08 mg of *o*-methyl- $\alpha$ -amanitin dissolved in 1.5 ml H<sub>2</sub>O. The mixture was stirred for 5 min at room temperature followed by reduction of the excess periodate by the dropwise addition of 1.1 ml of 0.09 *N* sodium bisulfite. This mixture was chromatographed on a column of Sephadex LH-20 (1.8  $\times$  100 cm) using H<sub>2</sub>O as the solvent.

Synthesis of *o*-methyl-[<sup>3</sup>H]-demethyl- $\gamma$ -amanitin was accomplished by reduction of

hyl-aldoamanitin with sodium borohydride (3). One and two tenths mg of m boro- $^{[3]H}$ -hydride (209 mCi/mg) was dissolved in 0.94 mg *o*-methyl-aldoamanitin dissolved in 1.5 ml of  $H_2O$ . The reaction mixture continuously stirred at  $0^\circ$  for 90 min at which time the mixture was acidified by the addition of 1 ml of 0.1 *N* HCl. After an additional 15 minutes the mixture was neutralized with 0.1 *N* NaOH. The reaction mixture was chromatographed on a Sephadex G-50 column using methanol/ $H_2O$  (1:1) as the solvent. Fractions found to have a proper uv-spectrum (4) and containing radioactivity were rechromatographed on a column of Sephadex G-50 ( $0.9 \times 15$  cm) in  $H_2O$  as the solvent. Peak fractions were identified by uv-absorbance and counting rates determined and the specific activity of hyl- $^{[3]H}$ -demethyl- $\gamma$ -amanitin was calculated. The specific activity was verified by comparison of the labeled derivative with purified wheat germ RNA polymerase in a binding assay described below.

**Thin layer chromatography of  $\alpha$ -amanitin derivatives.** In order to identify various reaction products and assess their purity, aliquots of peak fractions were concentrated and then studied by thin layer chromatography on Silica Gel-OF plates. Two solvent systems were employed, selected for their ability to separate the intermediate compounds. Chromatograms were visualized by staining with Erlich's solution or transcin-alddehyde/HCl (3, 5).  $\alpha$ -Amanitin was used as a reference standard against which migration of the intermediates was compared. Radioactive products were located by exposing 0.5 cm squares from moist plates to a thin layer chromatography. The resultant material was then digested overnight in Nuclear Chicago Solubilizer (NCS) at  $45^\circ$  and used in nonaqueous, toluene based scintillation.

**Infrared spectrophotometry of  $\alpha$ -amanitin and non-radioactive derivatives.** As an additional proof of the proper placement of functional groups in the amanitin molecule infrared spectra were obtained for  $\alpha$ -amanitin, *o*-methyl- $\alpha$ -amanitin and *o*-methyl-aldoamanitin. Small amounts of each compound (ca. 1 mg) were dissolved in  $H_2O$  and lyophilized. KBr pellets were prepared for each

sample using a Wilk's mini press. The pellets were scanned from 4000 to  $600\text{ cm}^{-1}$  on a Beckman Acculab 4 Infra-red Spectrophotometer. The reference beam was attenuated to permit adjustment of the baseline. Chromatographically pure  $\alpha$ -amanitin was used as a reference compound.

**Amatoxin competition binding assay.** The method used to demonstrate the binding of  $^{[3]H}$ - $\gamma$ -amanitin was based on the procedure of Cochet-Meilhac *et al.* (1, 2). Purified wheat germ RNA polymerase or the enzyme present in crude homogenates of baby mouse kidneys was used as a substrate for binding the radioactive ligand. Crude homogenates were prepared by grinding whole kidneys of baby mice (8-10d) in a Potter-Elvehjem tissue grinder in homogenizing buffer (50 mM Tris HCl pH 7.4; 0.1 mM EDTA; 0.1 mM dithiothreitol and glycerol 30% v/v). Aliquots of 100  $\mu$ l of the crude homogenates or 100  $\mu$ l of the purified enzyme in binding buffer ( $1.63 \times 10^{-8}$  M) were incubated in an assay mixture containing 500  $\mu$ l binding buffer (80 mM Tris HCl pH 7.9; 0.1 mM EDTA; 0.1 mM dithiothreitol; 150 mM  $(NH_4)_2SO_4$ ; 0.2 mg/ml bovine serum albumin; 0.4 mg/ml rabbit gamma globulin and 30% (v/v) glycerol), 10  $\mu$ l  $^{[3]H}$ - $\gamma$ -amanitin ( $9.13 \times 10^{-6}$   $\mu$ moles ca. 2.0 Ci/mmole) and 10  $\mu$ l of unlabeled  $\alpha$ -amanitin in varying concentrations. Controls for  $^{[3]H}$ - $\gamma$ -amanitin binding contained 10  $\mu$ l binding buffer in place of  $\alpha$ -amanitin. Samples were incubated at  $4^\circ$  for 18 hr. After 18 hr 1 vol of  $(NH_4)_2SO_4$  solution, saturated at  $4^\circ$ , was added to the reaction mixture and samples were kept at  $4^\circ$  for an additional hour. Free and unbound amanitin were then separated by centrifugation at 39,000g for 20 min. The supernatant was discarded and the pellet was redissolved in 1 ml of binding buffer; an equal volume of saturated  $(NH_4)_2SO_4$  was again added and the samples were incubated at  $4^\circ$  for 30 min, at which time they were recentrifuged as described above. This suspension-reprecipitation step was repeated two additional times. Finally the pellet was dissolved in 200  $\mu$ l of  $H_2O$ , digested overnight in NCS at  $45^\circ$  and counted in non-aqueous toluene based scintillant. Counting efficiency was approximately 85% of that obtained for unquenched samples. Values obtained with the highest

concentration of  $\alpha$ -amanitin were found to correspond to the background samples containing no RNA polymerase when the purified enzyme was used. When the assay was done using the crude homogenate as a source of RNA polymerase the values obtained at the highest concentrations of  $\alpha$ -amanitin were assumed to represent nonspecific binding of the labeled derivative. This value did not exceed 6% of the total label bound and was used to correct experimental values obtained with crude homogenates.

**[ $^3\text{H}$ ] Amanitin saturation assay.** To verify the specific activity of the [ $^3\text{H}$ ]- $\gamma$ -amanitin as determined by the ratio of radioactivity/absorbance at 310 nm an experiment was done to ascertain the amount of purified wheat germ RNA polymerase required to saturate a fixed amount of the radioactive ligand. Each sample contained [ $^3\text{H}$ ]- $\gamma$ -amanitin ( $1.47 \times 10^{-8}$  M) and variable concentrations of RNA polymerase from  $2.63 \times 10^{-9}$  M to  $5.26 \times 10^{-8}$  M. Concentration of [ $^3\text{H}$ ]- $\gamma$ -amanitin at saturation was based on the 50% end point. Conditions for the assay are identical to those described above for [ $^3\text{H}$ ]- $\gamma$ -amanitin binding controls.

**Materials.** The materials used for these experiments were obtained from the following suppliers: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *p*-dimethyl-aminobenzaldehyde and MNNG diazomethane generator, Aldrich Chemical Co. Milwaukee, WI;  $\alpha$ -Amanitin, Boehringer-Mannheim Biochemicals Indianapolis, IN; Nuclear Chicago Solubilizer (NCS) and sodium boro- $^3\text{H}$ -hydride, Amersham-Searle Co., Arlington Heights, IL; transcinnamaldehyde, Eastman Organic Chemical Rochester, NY; wheat germ RNA polymerase, Miles Laboratories, Elkhart, IN; Sephadex LH-20 and G-50, Pharmacia Fine Chemicals, Piscataway, NJ; Silica Gel OF TLC plates, New England Nuclear, Boston, MA; rabbit IgG and ultra pure ammonium sulfate Schwartz Mann, Orangeburg, NY; sodium periodate and sodium metabisulfate, Sigma Chemical Co., St. Louis, MO.

**Results. Synthesis of a radioactive derivative of  $\alpha$ -amanitin.** Chromatography of the methylation product of  $\alpha$ -amanitin on Sephadex LH-20 resulted in two peaks absorbing at 310 nm (Fig. 1). Thin layer chromatography of the material in fraction 13 in butanol/acetone/ $\text{H}_2\text{O}$  (30/3/5) yielded a band co-mi-

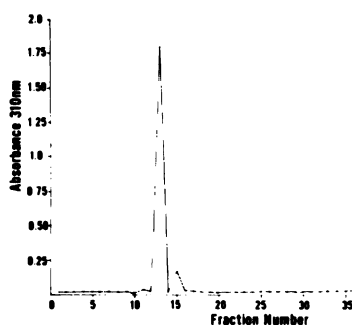


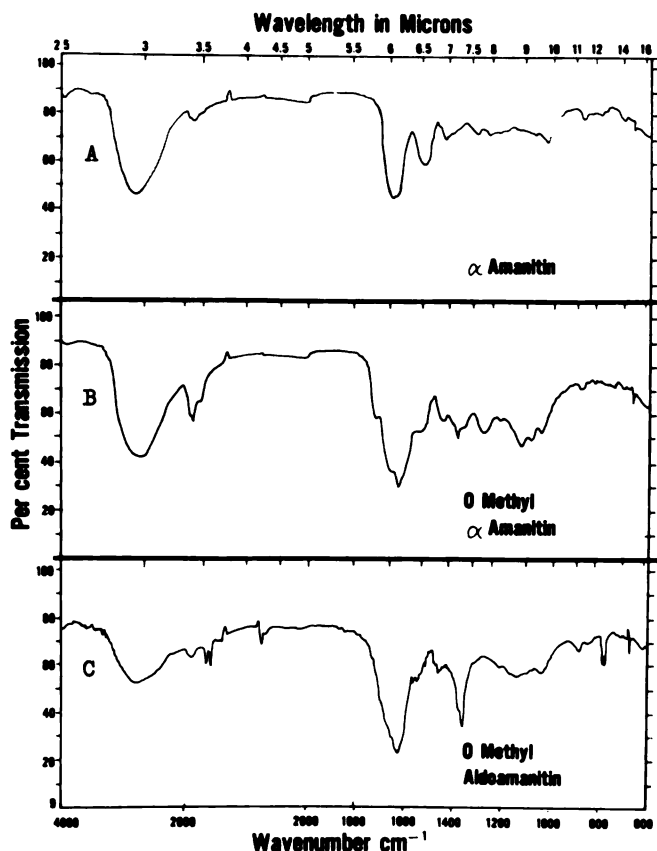
FIG. 1. Products of methylation of  $\alpha$ -amanitin chromatographed on a column of Sephadex LH-20 ( $100 \times 100$  cm) using methanol/ $\text{H}_2\text{O}$  1:1. The column was monitored at 310 nm. Fractions of 7.5 ml were collected at 40 min intervals.

grating with the  $\alpha$ -amanitin ( $R_f = 0.34$ ). With methanol/ $\text{H}_2\text{O}$  (4:1) we obtained an effective separation resulting in the  $\alpha$ -amanitin migrating further than the methylated derivative ( $R_f = 0.86$  vs. 0.34).

**Infra-red spectrophotometry.** The differences in the methylation product and the parent compound. The methylation product (an aryl phenolic hydroxyl group of the parent moiety) was expected to exhibit characteristic absorption in the regions of  $1300$ – $1180$   $\text{cm}^{-1}$  and  $1125$ – $1025$   $\text{cm}^{-1}$ . The comparison spectra of  $\alpha$ -amanitin and *o*-methyl- $\alpha$ -amanitin (Fig. 2) demonstrate changes between  $1300$ – $1180$   $\text{cm}^{-1}$  and  $1125$ – $1025$   $\text{cm}^{-1}$ .

The results of Sephadex LH-20 chromatography of the periodate oxidation product of *o*-methyl- $\alpha$ -amanitin are presented in Figure 3. Only the major peak was four fractions from the column. The typical uv-spectrum for an amatoxin layer chromatography using methanol/ $\text{H}_2\text{O}$  and stained with vanillin/ $\text{HCl}$  showed that the reaction product migrates slower than the  $\alpha$ -amanitin ( $R_f = 0.30$ ) and stains reddish brown rather than violet. Infra-red spectra showed changes at  $2800$   $\text{cm}^{-1}$  and  $1700$   $\text{cm}^{-1}$  consistent with the introduction of an aliphatic aldehyde group into the molecule (Fig. 2c).

Following reduction of *o*-methyl- $\alpha$ -amanitin with sodium boro- $^3\text{H}$ -hydride, the reaction products were separated on Sephadex LH-20. Three peaks absorbing at 310 nm were eluted (Fig. 4). The major leading peak did not possess a



A comparison of the infra-red spectra of  $\alpha$ -amanitin (2a), *o*-methyl- $\alpha$ -amanitin (2b) and *o*-methyl-aldo-amanitin (2c). Samples were prepared as KBr pellets and scanned at slow speed using the normal slit program of an Acculab 4 Infra-red Spectrophotometer. The reference beam was attenuated to obtain a suitable base line.

trum of an amatoxin and while the l in the center peak did resemble an in by uv spectrophotometry the rela-ount of incorporated radioactivity was w. Only the material in the trailing d both an amatoxin uv spectrum and icant amount of incorporated label. or peak of radioactivity was unasso-ith any amatoxin containing fraction s assumed to be unreacted. Fractions 40 were pooled, concentrated *in vacuo* -chromatographed on a column of ex G-50 to ensure complete removal unreacted radioactivity. The column is presented in Fig. 5. The major of radioactivity coincided with the uv-absorbance in fraction 11. Very itaminating radioactivity was present. of the material eluted from Sepha-0 was verified by thin layer chroma- y in butanol/acetone/H<sub>2</sub>O. The mi-

gration of the radioactive derivative was com- pared to the marker,  $\alpha$ -amanitin, which was detected by staining with Erlich's reagent. The results of the thin layer chromatography are presented in Fig. 6. The radioactive derivative migrates as a single band ( $R_f = 0.40$ ) ahead of the marker ( $R_f = 0.34$ ). Neither infra-red spectrophotometry nor destructive analytic techniques were employed to verify the chemical structure of the end product because of the small amount recovered. The end product is assumed to be *o*-methyl-[<sup>3</sup>H]-demethyl- $\gamma$ -amanitin since only the aldehyde formed in the previous step would be availa- ble for borohydride reduction.

*Saturation of wheat germ RNA polymerase with [<sup>3</sup>H]- $\gamma$ -amanitin.* The specific activity of [<sup>3</sup>H]- $\gamma$ -amanitin was determined by two in- dependent methods. Based on the uv absorp- tion and counting rates of several small sam- ples, the material in fraction 11 (Fig. 5) was

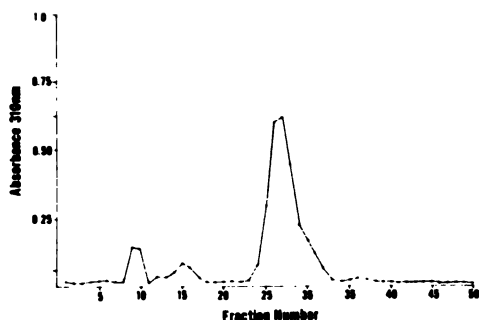


FIG. 3. Products of sodium periodate oxidation of *o*-methyl-aldoamanitin were chromatographed on a column of Sephadex LH-20 ( $1.8 \times 100$  cm) using  $H_2O$  as the solvent. Fractions contained approximately 6.25 ml. Column monitoring and fraction collection were accomplished as described in Fig. 1.

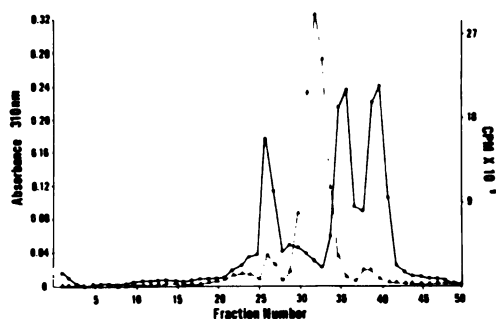


FIG. 4. The reaction products of sodium boro- $[^3H]$ -hydride reduction of *o*-methyl-aldoamanitin were chromatographed on a column of Sephadex LH-20 ( $1.8 \times 100$  cm) using methanol/ $H_2O$  (1:1) as the solvent. Flow rate, column monitoring and fraction size are described in Figure 1. 50  $\mu$ l aliquots of each fraction were used to approximate the total radioactivity. (●—●) absorbance 310 nm; ( $\Delta$ — $\Delta$ )  $[^3H]$  cpm.

estimated to contain  $7.25 \times 10^{-3}$   $\mu$ moles/ml *o*-methyl- $[^3H]$ -demethyl- $\gamma$ -amanitin with a specific activity of 2.50 Ci/mmol. Fraction 12 was found to contain  $7.23 \times 10^{-3}$   $\mu$ moles/ml, and have a specific activity of 2.16 Ci/mmol. Specific activity estimates based on the saturation of  $[^3H]$ - $\gamma$ -amanitin with wheat germ RNA polymerase agreed well with those obtained by instrumental methods. The fifty percent maximum binding of RNA polymerase was found to occur at  $7.35 \times 10^{-9}$   $M$ . Assuming that the reaction was at equilibrium, had a very small  $K_D$  (approximately  $10^{-11}$   $M$ , see ref. 1, 2) and that the purified enzyme contained a single binding site, the concentration of  $[^3H]$ - $\gamma$ -amanitin

was calculated to be  $1.47 \times 10^{-5}$   $M$  and the specific activity 1.88 Ci/mmol. Saturation data are presented in Fig. 7.

**Amatoxin competition assay.** A competition assay was designed to test the hypothesis that  $[^3H]$ - $\gamma$ -amanitin bound to the same site as  $\alpha$ -amanitin and with approximately the same affinity. The concentration of the radioactive derivative was constant at  $1.47 \times 10^{-8}$   $M$  (approximately 12,000 cpm) and the concentration of the competing, unlabeled  $\alpha$ -amanitin was varied from  $8.27 \times 10^{-11}$  to  $2.62 \times 10^{-5}$   $M$ . The assays were carried out at four to five times the concentration of  $[^3H]$ - $\gamma$ -amanitin required to saturate the amount of RNA polymerase present. Controls for non-

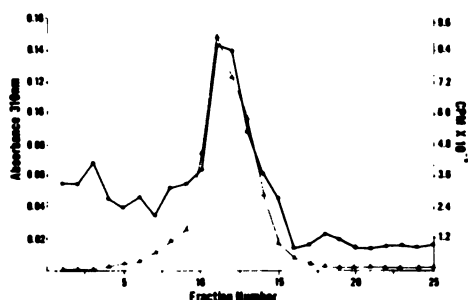


FIG. 5. *o*-methyl- $[^3H]$ -demethyl- $\gamma$ -amanitin contained in fraction 39 (Fig. 4) was chromatographed on a column of Sephadex G-50 ( $0.9 \times 15$  cm) using  $H_2O$  as the solvent. Each fraction contains 0.75 ml; flow rate 0.38 ml/min. Five microliter aliquots of each fraction were assayed for radioactivity (●—●) absorbance 310 nm; ( $\Delta$ — $\Delta$ )  $[^3H]$  cpm.

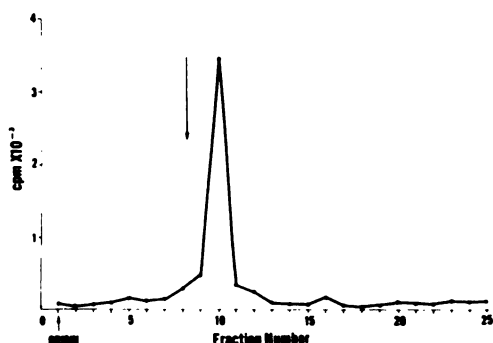
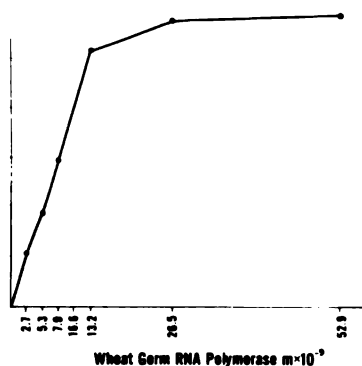


FIG. 6. Thin layer chromatography of *o*-methyl- $[^3H]$ -demethyl- $\gamma$ -amanitin. A 5  $\mu$ l aliquot of the peak fraction eluted from Sephadex G-50 was chromatographed in butanol/acetone/ $H_2O$  (30:3:5). Migration of  $\alpha$ -amanitin indicated by arrow. Each point represents a migration of 0.5 cm.



7. Saturation of wheat germ RNA polymerase with *o*-methyl- $^{[3]H}$ -demethyl- $\gamma$ -amanitin. *o*-methyl- $\gamma$ -amanitin ( $1.47 \times 10^{-8} M$ ) was incubated for 18 hr in the presence of increasing concentrations of wheat germ RNA polymerase ( $2.63 \times 10^{-9} M$  to  $32.9 \times 10^{-9} M$ ). Assay mixture was the same as used for the competition assay except unlabeled  $\alpha$ -amanitin was omitted.

ic binding did not contain the enzyme bound less than 0.8% of the total input. The results of competition assays for both purified wheat germ RNA polymerase and enzyme present in crude homogenates are plotted in Fig. 8. The percent bound  $^{[3]H}$ -amanitin was determined by calculating the ratio of counts bound for each concentration of  $\alpha$ -amanitin to the counts bound in samples containing no unlabeled competing amanitin. The ideal curve is based on the decrease in specific activity of the total amanitin concentration at saturation, assuming a single binding species is present and both compounds compete equally for the binding site. The experimental results for enzyme preparations closely approximate the ideal curve.

**Discussion.** Previous studies have demonstrated *o*-methyl- $^{[3]H}$ -demethyl- $\gamma$ -amanitin as a powerful tool in studying the eukaryotic nucleoplasmic RNA polymerase (1, 2). However, the unavailability of this compound or of large enough amounts of  $\alpha$ -amanitin to synthesize this derivative by the previously reported method has restricted the widespread application of this technique.

The procedures presented in this paper permit the synthesis of small amounts of  $\gamma$ -amanitin from readily available quantities of starting materials by the introduction of nondestructive analytic techniques to

ensure proper placement of functional groups in nonradioactive intermediates. Use of shorter columns of Sephadex LH-20 did not affect the desired resolution and the introduction of a short column of Sephadex G-50 ensures complete removal of any unreacted radioactivity in the end product. This is verified by thin layer chromatography of the radioactive product and further substantiated by the saturation curve of  $^{[3]H}$ - $\gamma$ -amanitin with purified wheat germ RNA polymerase B in which less than 3% of the total input remained unbound. The competition assay conclusively demonstrates that the final product binds to RNA polymerase in essentially the same manner as the unreacted parent compound and at the same site.

Although the competition assay was designed as a test for reactivity and radiochemical purity of the final product, the usefulness of this assay exceeds this purpose. Currently, studies of amanitin resistant RNA polymerase B have relied on the inhibition of enzyme activity by various concentrations of amatoxin to characterize wild type or mutant enzymes (6, 7). This technique cannot be applied to crude cell homogenates as the normally resistant RNA polymerases A and C as well as RNase would complicate the kinetic analysis.

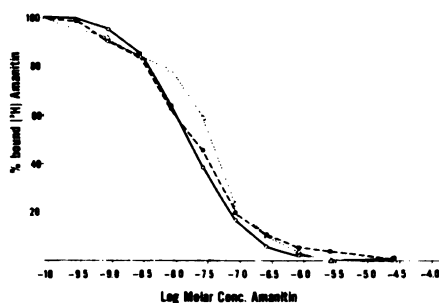


FIG. 8. Competition binding assay. The ideal curve (O—O) represents the percent of the total amatoxin present as *o*-methyl- $^{[3]H}$ -demethyl- $\gamma$ -amanitin. The assay was carried out in the presence of a constant amount of *o*-methyl- $^{[3]H}$ -demethyl- $\gamma$ -amanitin ( $1.47 \times 10^{-8} M$ ) previously determined to be in excess required to saturate either the purified wheat germ RNA polymerase ( $2.63 \times 10^{-9} M$ ) or the enzyme present in 0.0189 g of mouse kidney homogenate. Percent saturation was calculated from the total radioactivity bound in samples containing no unlabeled  $\alpha$ -amanitin; wheat germ RNA polymerase ( $\Delta \cdots \Delta$ ); crude mouse kidney homogenate ( $\bullet \cdots \bullet$ ).



The amanitin competition assay could provide a new method for studying the interaction of amanitin with resistant RNA polymerase B enzymes. The assay is essentially free from interference by other RNA polymerases and is unaffected by RNase, therefore, crude homogenates as well as purified enzyme preparations can be studied. In addition, the assay provides a means of direct measurement of dose-response over a wide range of concentration and could provide additional insight into possible mechanisms of amanitin resistance.

**Summary.** An improved method permitting the synthesis of a radioactive derivative of  $\alpha$ -amanitin from a small amount of the commercially available parent compound has been developed. The labeled derivative was used in an amatoxin competition binding assay designed to detect eukaryotic RNA polymerase B in either purified form or in crude homogenates. Both compounds are shown to compete for the same binding site and with approximately the same affinity. The competition assay proves to be both sensitive and highly selective for RNA polymerase B and

provides a new, direct method for studying the enzyme-amanitin interaction over a much broader range of concentration previously reported.

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## Exometabolites of *Leishmania donovani* Promastigotes. I. Isolation and Initial Characterization (40293)<sup>1</sup>

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Isolation and characterization of parasitic exometabolites is of importance in understanding the possible role these may play in the host-parasite relationship. Since the products of intracellular parasites do become host cell contaminated with host cell substances, isolation, purification, and characterization of these parasite products becomes

*Leishmania* provides an ideal system where the release of exometabolites produced by an active intracellular protozoan parasite from its host may readily be studied. *Leishmania* organisms have two morphological forms: the amastigote, an obligative intracellular form infecting vertebrates, and the promastigote, which exists extracellularly in the environment and will grow readily in culture. Promastigotes have been reported to produce various exometabolites which demonstrate immunogenic identity (1, 2).

Experiments to date dealing with substances released into the media in which promastigotes are metabolizing (metabolized media) have involved either undefined media containing blood proteins (1, 2) or salt solutions (4, 5). In order to determine if promastigote substances were present in the media, Senekji's medium after promastigote growth, Clinton *et al.* (3) utilized electrophoretic procedures, reacting the metabolized medium against antiserum from rabbits to the homologous promastigotes. One band formed between the antiserum and a substance from the metabolized

medium. No reaction was observed when nonmetabolized medium was tested. Schnur *et al.* (1) utilized metabolized Feinberg and Whittington's medium and reacted this with rabbit antipromastigote hyperimmune serum and demonstrated multiple bands (termed EF) by diffusion in gels. Since Schnur *et al.* obtained their metabolized medium from cultures of promastigotes in log phase, they concluded that the EF substances were exometabolites and not products of lysis. In addition, the molecular weight of the EF substances was within the range of 25,000 and 70,000, but they were not immunogenic when injected into rabbits. Decker and Honigberg (6), however, reported successful induction of antibodies in mice to the exometabolite. Results utilizing less defined media suggest that promastigotes of *Leishmania* produce exometabolites, but there is no agreement as to their number and immunogenicity (1, 3, 6). The lack of agreement in the data may be attributed to the different media used to culture the promastigotes.

Media used in *in vitro* culture should be defined and protein free to facilitate recovery of exometabolites more closely resembling the native form released from the parasite. Greenblatt and Glaser (4) used Locke's solution with glucose at 37° to maintain promastigotes and found a variety of molecules including various amino acids, hypoxanthine, guanosine, uracil, and ribose in the medium. They did not detect any large molecules and concluded that the low molecular weight substances found in the metabolized medium resulted from leakage and not gross lysis. On the other hand, Decker and Janovy (5) in a similar study detected not only small molecules but also proteins and RNA. Thus, while salt solutions may be ideal for recovery of leakage products from promastigotes, they may not adequately support complete metabolism of the organisms. Measurable quantities of larger molecular weight excretion-

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secretion metabolic products may not accumulate. On the other hand, the higher molecular weight products detected could be the result of lysis.

More recently Slutzky and Greenblatt (7) isolated a substance by degradative isolation techniques (boiling and 33% trichloroacetic acid solution) from proteid *L. tropica* metabolized medium. The substance isolated was initially associated with medium protein, was immunologically active and carbohydrate rich. The isolated entity did not pass through a 30,000 mol wt exclusion membrane. Little or no protein was reported to be associated with the isolated entity.

The object of this study was to isolate and characterize the metabolic by-products of *L. donovani* promastigotes in their native form. To accomplish this log phase promastigotes of *L. donovani* were maintained in protein free tissue culture medium to minimize the interference of lytic by-products. The metabolized medium was then fractionated and examined spectrophotometrically and serologically.

**Materials and methods.** Amastigotes utilized to initiate promastigote cultures were obtained from the spleens of hamsters infected with the 3S strain of *L. donovani* (8). Spleens were homogenized in sterile phosphate-buffered (pH 7.0) physiological saline and amastigotes isolated by differential centrifugation (9). All cultures were initiated at a density of  $5 \times 10^4$  organisms per ml and subcultures were made when the density of a culture reached  $2 \times 10^7$  promastigotes per ml. Promastigotes utilized to generate metabolized culture medium were never less than 4 nor more than 15 subcultures removed from the initial amastigote-seeded culture. All cultures were incubated with an atmosphere of 5% CO<sub>2</sub> in air at  $25 \pm 0.1^\circ$ .

The culture medium utilized to grow promastigotes (growth medium) consisted of 9 parts Medium 199 with Hanks' salts (Gibco) and 1 part whole defibrinated rabbit blood (Pel Freeze). The blood was centrifuged (4 hr at 2000g) before inclusion into the medium to separate serum from cells. Serum was inactivated at  $56^\circ$  for 30 min and stored at  $-20^\circ$  until used. Cells were washed in excess Hanks' balanced salt solution (Gibco) 5 times and lysed in a volume of double distilled

water equal to 10 $\times$  the packed cell vol. Cell ghosts were removed by centrifuging (24 hr at 200g) and the supernatant utilized in the medium. To prepare 1 liter of growth medium, 100 ml of Medium 100 (10 $\times$ ) was added slowly to 500 ml of lysate and a sufficient amount of double distilled water added to bring the volume to 950 ml. The pH was maintained at 7.2 by addition of NaHCO<sub>3</sub> as needed. Serum (50 ml) was then added and the medium sterilized by filtration through a 0.22  $\mu$ Millipore filter.

Medium used to maintain promastigotes (maintenance medium) consisted of Medium 199 with Hanks' salts (Gibco) and 25 mM Hepes buffer (Sigma). The pH was adjusted to 7.2 with 1 N HCl or NaOH. The maintenance medium was sterilized as described above.

Promastigotes were allowed to metabolize both growth and maintenance media. Growth medium cultures were initiated at a density of  $5 \times 10^4$  organisms per ml. When promastigote density reached  $8 \times 10^6$  per ml (mid log phase), the cultures were centrifuged (20 min at 2000g) separating promastigotes from the medium. Organisms were washed 3 times in excess Hanks' balanced salt solution and resuspended in maintenance medium at a density of  $10^7$  promastigotes per ml. Cultures in maintenance medium were incubated 8 hr at  $25^\circ$  with a 5% CO<sub>2</sub> in air atmosphere. Promastigotes were removed by centrifugation (1 hr at 2000g) and the metabolized medium was filtered through a 0.22  $\mu$ Millipore filter, concentrated 10 $\times$  by lyophilization and stored at  $-20^\circ$ .

Two ml aliquots of 10 $\times$  concentrated metabolized maintenance medium were fractionated on a column (1.6  $\times$  80 cm) of fine grade Sephadex G25 (Pharmacia). The column volume was 54 ml, bed volume was 10 ml and flow rate was 7 ml per hr. The eluent used was a 5% acetic acid solution in distilled water. The column was characterized using  $\alpha$ -melanocyte stimulating hormone (mol wt 1910; Bradykinin, mol wt 1204; and glycylpentapeptide, mol wt 768, all purchased from Calbiochem. Elution values were 94, 120 and 145 ml respectively. Each standard was applied to the column as a 1 ml volume containing 50  $\mu$ g peptide. Elution volume was determined from the maximum of the elution

ilution values for the standards against the log of their molecular approximate a straight line.

nl fractions were collected from the and analyzed on a Beckman DB 24 spectrophotometer. Absorption were obtained between 190 and 350

mount of peptide present in a fraction nated photometrically by the method er and Miller (10). Fractions were ed to dryness and redissolved in sol- 15 M NaF in glass double distilled The blank contained solvent only. nce was measured at 193 nm and a l curve generated using bovine serum ,  $\alpha$ -melanocyte stimulating hormone, pentapeptide and Bradykinin (Calbi-

The standard curve developed here stinguishable from that presented by and Miller with 11  $\mu$ g/ml protein an absorbance value of 0.7. Direct onality between concentration and nce was applicable for all standards absorbance of 0.7. Since all fractions 1 protein amount was estimated had nce values greater than 0.7, aliquots ons were diluted with solvent until an nce value of 0.7 was attained. The of protein in a fraction was calculated plying the dilution factor  $\times$  11  $\mu$ g/ml. amount of sugar present in fractions ermined by the procedure of Dubois 1). A standard curve for D-galactose erated which was indistinguishable at presented by Dubois *et al.* The value for triplicate samples contain- g of D-galactose was 0.11 absorbance 490 nm.

fic chemical tests for tryptophan and were performed on selected fractions procedure of Fischl (12) and the of Udenfriend and Cooper (13) as d by Massin and Lindenberg (14) re- ly. Controls were composed of 50 olutions of tryptophan, tyrosine and

*L. donovani* promastigote immune se- s raised in rabbits by injecting a ho- e composed of freeze-thawed pro- es in saline and FCA (1:1). Each ceived a total volume of 1 ml, con- 21 mg N (determined by Kjeldahl

procedure [Campbell *et al.*, 15]) delivered in 0.1 ml aliquots at one time to 8 sc sites on the back and 2 im sites in the hind legs. The animals were bled 30 days after immuniza- tion. Serum was recovered by centrifugation (1 hr at 2000g) and stored at  $-20^{\circ}$ .

Test antigens were prepared by mixing (6:1) metabolized maintenance medium (free of serum) with nonmetabolized growth medium (containing serum) and concentrating tenfold by lyophilization. Control antigens were nonmetabolized growth medium and nonmetabolized maintenance medium pre- pared in the same manner.

The microsolute in each sample were ex- changed by diafiltration (16) and standard- ized using a 500 mol wt cutoff ultrafiltration membrane (UM 05) with a Model 12 stirred cell (Amicon). Five sample volumes of bar- bital buffer (17) were exchanged with a pre- dicted 99+% complete exchange of micro- solute (16).

Gel diffusion plates were prepared by pouring 10 ml melted agar solution (1% Difco Bacto Agar in barbital buffer [17] with 0.1% sodium azide) into a 9 cm-diameter petri dish. Wells (5 mm O.D.) were cut in the agar 7.5 mm apart (center to center). After the wells were filled with either antiserum or antigen solution, the plates were incubated 48 hr in a humid atmosphere at  $25^{\circ}$ . Precipitin lines appeared within 1 to 2 days but were allowed to develop for a total of 4 to 7 days. Gels were washed free of nonreacting protein with barbital buffer (17) for 48 hr (4 changes of buffer) and stained wet with a saturated so- lution of picric acid in 1% acetic acid.

**Results.** When promastigote metabolized and nonmetabolized growth media were tested against rabbit antipromastigote im- mune serum by gel diffusion, the metabolized medium reacted forming multiple precipitate bands. This confirmed earlier reports that exometabolites were present in the metabo- lized growth medium and would react with specific antiserum (1, 3). When promastigote metabolized maintenance medium was tested against the same antiserum, no reaction oc- curred. This suggested that the presence of serum protein was necessary for the exome- tabolite to react with antibody.

To determine whether serum protein was indeed essential for formation of specific pre-

cipitates, metabolized and nonmetabolized maintenance media were mixed with non-metabolized growth medium, the microsoluble environment standardized, and reacted with immune serum. The mixture containing metabolized maintenance medium yielded multiple precipitate bands identical to the ones observed when metabolized growth medium was used as the reacting antigen (Fig. 1). No reaction occurred with the nonmetabolized medium.

When metabolized maintenance medium was fractionated, spectrophotometric analysis at 274 nm revealed two major fractions (A and B) (Fig. 2) with elution values of 101 and 122 ml respectively. Ultraviolet absorption spectra of these major fractions from 190 to 350 nm are shown in Fig. 3. None of the fractions from nonmetabolized maintenance medium demonstrated either of the major peaks shown in Fig. 2.

When all fractions collected after column chromatography of either metabolized maintenance medium or nonmetabolized maintenance medium were mixed with nonmetabolized growth medium and tested against antipromastigote immune serum, only Fraction B reacted to form precipitate bands (Fig. 1). These precipitate bands demonstrated reactions of identity with those formed against antipromastigote immune serum using promastigote metabolized growth medium as the



FIG. 1. Gel diffusion plate depicting reactions of promastigote metabolized growth medium (well A), concentrated promastigote metabolized maintenance medium mixed with nonmetabolized growth medium (well B), concentrated promastigote metabolized maintenance medium (well C) and concentrated nonmetabolized maintenance medium mixed with nonmetabolized growth medium (well D) against rabbit antipromastigote immune serum (well E).

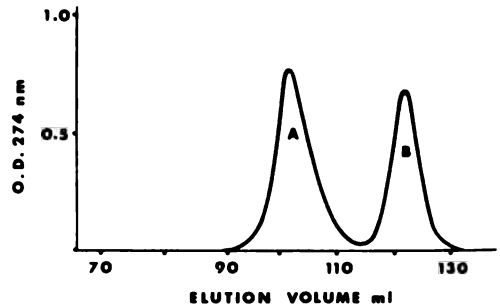


FIG. 2. Sephadex G25 gel filtration profile of metabolized maintenance medium at 274 nm.

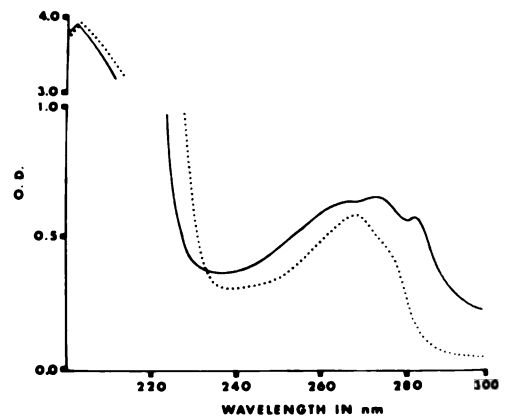


FIG. 3. Ultraviolet absorption spectra of gel filtration Fraction A (-----) and Fraction B (—), pH 7.2.

reacting antigen. When Fraction A (10 absorbance units) was mixed with the antiserum prior to reaction with Fraction B, no evidence of neutralization was observed.

Fraction A and B samples with absorption values of 3.1 (at 293 nm) were estimated to contain approximately 48  $\mu$ g peptide and 10  $\mu$ g sugar per ml. Fractions from the column which eluted both immediately before and after Fractions A and B were determined not to contain sugar.

**Discussion.** The results suggest that at least two low molecular weight substances are recoverable from promastigote metabolized protein free medium during the log phase growth of the organisms. No high molecular weight substances were detected as might have been anticipated if the recovered substances were the result of promastigote lysis. Microscopic examination of log phase cultures revealed no lysed organisms suggesting that recovered substances are indeed exometabolites and not products of autolysis. Spec-

ita shown in Fig. 3 suggest the presence  
ide bonds (10) with tyrosine present in  
on A and tryptophan in Fraction B  
The presence of these amino acid resi-  
was confirmed by colorimetric proce-

Detection of sugar in Fractions A and  
gests that the substances may be glyco-  
les. Since the molecules appear to be of  
olecular weight and the ratio of protein  
ar is approximately 5:1, the carbohy-  
entity is most likely composed of only  
units.

olecular weights of the substances in  
ons A and B appear to be in the range  
1-1900 in that their elution values were  
mediate between those of gastrin penta-  
le (mol wt 768) and  $\alpha$ -melanocyte stim-  
g hormone (mol wt 1911) (see Andrews  
It is premature at this time to assign a  
precise molecular weight. The estimated  
ular weight of recovered substances  
sts glycopeptides composed of from 5 to  
ino acid residues. Peptides of this size  
be expected to act as simple haptens

erally low molecular weight substances  
t induce an imune response unless con-  
d to a larger carrier molecule (21). The  
gation of low molecular weight material  
rotein carrier endows that conjugated  
n with multivalency with respect to the  
nic moiety (20). The exometabolites ap-  
o act as monovalent haptenic groups.  
ata suggest that the simple substance  
ed in Fraction B attaches to sites on the  
n molecule making the conjugated mol-  
multivalent with respect to that site and  
ble to form precipitates when reacted  
antipromastigote immune serum. This  
retation is supported by the fact that  
nds formed with the promastigote me-  
zed growth medium are identical to  
observed when the substance in Frac-  
t is mixed with protein. The substance  
ction A did not form precipitates when  
against antipromastigote immune se-  
This may have occurred because no  
alent entities formed or because there  
isufficient antibody present specific for  
oiety.

exometabolite produced by *L. tropica*  
en reported to be a carbohydrate-rich  
nce that does not pass through a 30,000

mol wt exclusion membrane (7); however, it  
has been demonstrated to be adsorbed ini-  
tially to medium proteins. While it is not  
impossible that *L. donovani* and *L. tropica*  
produce physically distinct exometabolites,  
the major differences reported may result  
from the method of isolation. The *L. donovani*  
exometabolite reported here was isolated by  
gentle procedures under mild conditions  
while Slutzky and Greenblatt utilized more  
harsh procedures.

Fraction B exometabolite is released by  
both amastigotes and promastigotes as evi-  
denced by the fact that reactions of identity  
occur when promastigote metabolized growth  
medium and amastigote infected spleen ho-  
mogenate supernatant react with antipromas-  
tigote immune serum (2). *Leishmania dono-*  
*vani* promastigote metabolized growth me-  
dium has been used as a vaccine and induced  
specific protection against amastigote chal-  
lenge (22). If the protective substance in me-  
tabolized medium is a conjugated antigen,  
then Fraction B exometabolite may be the  
antigenic determinant responsible for the  
protection. Work is proceeding to determine  
if Fraction B, after conjugation to a protein  
carrier, will act as an immunogen and induce  
specific protection.

**Summary.** Two exometabolites have been  
demonstrated to accumulate in protein free  
culture medium in which log phase promas-  
tigotes of *L. donovani* are metabolizing. These  
molecules demonstrate gel filtration charac-  
teristics suggesting a molecular weight in the  
range of 800-1900. The ultraviolet absorption  
spectra of the exometabolites suggest the  
presence of peptide bonds with tyrosine pres-  
ent in one and tryptophan in the other. Sugar  
was demonstrated to be associated with both  
Fractions A and B, suggesting the exome-  
tabolites are glycopeptides. The exometabo-  
lite in Fraction B did not react with specific  
antibody to form precipitates unless it was in  
combination with serum protein. The data  
strongly suggest that the exometabolite con-  
jugates with protein forming a multivalent  
entity.

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# Formation of Acyclic and Cyclic *N*-Nitrosamines by Cultured Human Colon<sup>1</sup> (40294)

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So compounds are a major class of carcinogens which are candidates to cause human cancers (1). *N*-Nitrosamines are detected in ambient air over certain areas (2), in tobacco smoke (3), in foods (4, 5). Furthermore, they can be formed *in vivo* by the reaction of nitrite with secondary amines under acid conditions, in the stomach (6). They may also be produced by enteric bacteria e.g. *E. coli in situ*. *N*-Nitrosamines require metabolic activation to exert their mutagenic and carcinogenic activity (8-10). This requirement could, in part, explain the organo-specificity of the *N*-nitrosamines. Furthermore, they could affect an individual's susceptibility to the carcinogenic action of other amines.

Experimental systems to study carcinogenesis in human epithelia are being developed (11, 12). We have previously reported that cultured human colon can activate carcinogens from several chemical classes: polycyclic aromatic hydrocarbons, hydrazine, *N*-nitrosamines, into carcinogens which bind to cellular macromolecules (13). We now report metabolic activation of several aliphatic *N*-nitrosamines in human colonic mucosa.

**Materials and methods.** Non-tumorous human colonic tissues were collected at the time of surgery or "immediate" autopsy (14) from 11 patients; 7 with and 4 without cancer of the colon. The tissues were quickly put in sterile containers on ice and submerged in L-15 medium within 15 min after removal from the patient and kept at 4° until cultured. The specimens were cut into squares (0.5 × 0.5 cm) and cultured as previously described (13).

After 24 hrs in culture, one of the following

[<sup>14</sup>C]labeled *N*-nitrosamines (New England Nuclear, Boston, MA) was added to the culture media to give a concentration of 100 μM: [<sup>14</sup>C]Dimethylnitrosamine [35 mCi/mmole; prepared on NCI contract N01-CP-55677 and purified by the method of den Engelse *et al.* (15); *N*-[<sup>14</sup>C-1-ethyl]diethylnitrosamine (14.5 mCi/mmole); *N*-[<sup>14</sup>C-2,6]nitrosopiperidine (18.8 mCi/mmole); *N*-[<sup>14</sup>C-2,5]nitrosopyrrolidine (16.2 mCi/mmole); *N*-[<sup>3</sup>H-3,4]nitrosopyrrolidine (5 mCi/mmole). *N,N'*-[<sup>14</sup>C(U)]dinitrosopiperazine (16.5 mCi/mole); *N*-[pyrrolidine-<sup>14</sup>C-2] nitrososornicotine (4.10 mCi/mmole).

Five explants per experimental variable in three sterile 60 mm plastic Petri dishes (Falcon Plastics, Oxnard, CA) were placed on a rack in a closed container (Nalgene plastic jar, 500 ml) which was modified with two ports for replacing air with 95% O<sub>2</sub>-5% CO<sub>2</sub> (16). The containers were placed on a rocker platform and rocked approximately 10 cycles per minute for 24 hr. In order to remove <sup>14</sup>C-CO<sub>2</sub> formed by the metabolism of the *N*-nitrosamine the containers were flushed with N<sub>2</sub> for 5 min and the CO<sub>2</sub> absorbed in two tubes each containing 8 ml 0.2 M Ba(OH)<sub>2</sub>. After removal of the explants, 1 ml 3M phosphoric acid (pH 3) was added to each culture dish to release CO<sub>2</sub> dissolved in the media. After 4 hr at 37°, the containers were then flushed with N<sub>2</sub> for another 5 min.

The tissue culture medium was transferred to a reaction flask (Kontes Glassware, Vineland, NJ) the sidearm of which contained a small vial with 0.5 ml 4N KOH, and oxidized by HgCl<sub>2</sub> (100 mg/ml) at 90° for 1 hr (15). The KOH-solution was added to the Ba(OH)<sub>2</sub>-solution. The precipitate was collected on Whatman GF/C filters and washed with absolute ethanol until the count in the washing solution was negligible. Medium without explants of colon served as control. The precipitate and filter were suspended in

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3 ml water and 10 ml Aquasol liquid scintillation cocktail (New England Nuclear, Boston, MA) and counted.

The mucosa was scraped from the explant, and DNA and protein isolated by the phenol extraction procedure. DNA was purified on a CsCl-gradient and the binding level measured as previously described (17). Binding to protein was also assayed (13). One explant from each variable was fixed in 3% glutaraldehyde buffered with 0.1 M *s*-collidine (pH 7.4) and prepared for light microscopy (18).

DNA, isolated from a total of 54 explants (pooled from three cases), was hydrolyzed with 0.1 M HCl at 70° for 1 hr and bases were isolated by high-pressure liquid chromatography (Column: Durrum DC 1-A; 15 × 0.21 cm; Durrum Chemicals, Sunnyvale, CA; Solvent: 0.1 M ammonium formate, pH 4.5; Flow rate: 0.6 ml/min). Markers for N-7 and O-6 methylguanine were added to the hydrolyzed DNA; the elution was monitored at 254 mM and 0.4 ml fractions were col-

lected. The radioactivity was measured by liquid scintillation methods. The material eluting in the void volume (90% of the radioactivity) was treated with conc. perchloric acid at 100° for 1 hr and methanol removed by vacuum-distillation and the radioactivity was determined.

**Results.** Formation of  $^{14}\text{C}$ -CO<sub>2</sub> after incubation of *N*-nitrosamines with human colon indicates that cultured human colonic mucosa is able to metabolize both acyclic *N*-nitrosamines (Table I), such as dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), and cyclic *N*-nitrosamines (Table II). Variation in the ability to metabolize cyclic *N*-nitrosamine was observed among individuals. Under these test conditions only *N*-nitrosopyrrolidine (NPy) was metabolized by all cases studied, *N,N*-dinitrosopiperazine (DNP) by five cases and *N*-nitrosopiperidine only by one case. No  $^{14}\text{C}$ -CO<sub>2</sub> was formed from *N*-nitrososornicotine possibly due to the chemical structure (the C-14 labeled atom

TABLE I. METABOLISM OF *N,N*-DIALKYL NITROSAMINES BY CULTURED HUMAN COLON.<sup>a</sup>

Case	Dimethylnitrosamine			Diethylnitrosamine		
	DNA <sup>b</sup>	Protein <sup>b</sup>	CO <sub>2</sub> -formation <sup>c</sup>	DNA <sup>b</sup>	Protein <sup>b</sup>	CO <sub>2</sub> -formation <sup>c</sup>
62	570	106	6920	N.D. <sup>d</sup>	22	6632
66	36	59	1381	N.D.	26	N.D.
83	12	29	566	N.D.	55	93
87	23	49	823	26	11	217
92	50	178	1040	N.D.	14	N.D.
99	29	133	849	N.D.	18	N.D.

<sup>a</sup> Colonic explants were cultured in chemically defined media for 24 hrs and the [ $^{14}\text{C}$ ]labelled *N*-nitrosamines were added at a concentration of 100  $\mu\text{M}$  to groups of five explants for 24 hr.

<sup>b</sup> pmoles nitrosamine bound per mg of either DNA or protein, single determination.

<sup>c</sup> pmoles  $^{14}\text{C}$ -CO<sub>2</sub> formed per mg DNA.

<sup>d</sup> N.D. = not detectable.

TABLE II. METABOLISM OF CYCLIC *N*-NITROSAMINES BY CULTURED HUMAN COLON.<sup>a</sup>

Case	<i>N</i> -nitrosopyrrolidine			<i>N</i> -nitrososornicotine			<i>N</i> -nitrosopiperidine			<i>N</i> -nitrosopiperazine		
	DNA	Protein	CO <sub>2</sub> -formation <sup>c</sup>	DNA	Protein	CO <sub>2</sub> -formation <sup>c</sup>	DNA	Protein	CO <sub>2</sub> -formation <sup>c</sup>	DNA <sup>a</sup>	Protein	CO <sub>2</sub> -formation <sup>c</sup>
62	55	56	2410	N.D. <sup>c</sup>	15	N.D.	N.D.	23	188	N.D.	185	9531
66	21	49	4276	N.D.	17	N.D.	N.D.	N.D.	N.D.	N.D.	216	N.D.
83	13	125	1190	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	15	596
87	103	51	478	22	N.D.	N.D.	N.D.	N.D.	N.D.	15	169	520
92	22	80	479	N.D.	7	N.D.	N.D.	21	N.D.	N.D.	206	1344
99	12	147	1056				N.D.	40	N.D.	N.D.	227	591
105 <sup>d</sup>	71	5910										
111 <sup>d</sup>	86	18,220										
114 <sup>d</sup>	99	6776										

<sup>a</sup> Colonic explants were cultured in chemically defined media for 24 hr and the [ $^{14}\text{C}$ ]labelled *N*-nitrosamines were added at a concentration of 100  $\mu\text{M}$  to groups of five explants for 24 hr.

<sup>b</sup> dpm per 100  $\mu\text{g}$  of either DNA or protein, single determination.

<sup>c</sup> pmoles  $^{14}\text{C}$ -CO<sub>2</sub> formed per mg DNA.

<sup>d</sup> Incubated.

<sup>e</sup> N.D. = none detectable.

ly one C-H bond)—but nonlabeled adducts have been formed from other carcinogens in the pyrrolidine ring. Only DMN and NPy consistently formed alkylated adducts which reacted with cellular DNA and protein. DMN, DEN, NPy, and DNP all formed adducts with cellular DNA and protein; when compared to the other nitrosamines high binding levels of DNP to cellular protein were observed. The binding data in Table II is given as either dpm per 100 µg DNA or dpm per 100 µg protein. The exact chemical structure of the adducts

between the *N*-nitrosamines and the nucleic acids are unknown at the present. A positive correlation ( $r = 1.00$ ) was found between the alkylation of DNA by DMN and DEN, while NPy did not show any correlation ( $r = 0.24, p > 0.1$ ). No correlation was found between DMN and NPy binding to protein and  $\text{CO}_2$ -formation was found ( $r = 0.14, p > 0.1$  and  $r = 0.41, p > 0.1$ , respectively). The alkylated DNA in both N-7 and O-6 positions of guanine (Table III). However, only the radioactivity was associated with the initial peak. Treatment of this material with conc. perchloric acid released  $\text{H}^+$  (40% of radioactivity). The morphology of the explants, as monitored by high resolution light microscopy, showed good morphology in all the reported cases.

**Discussion.** *N*-Nitroso compounds induce cancer in many animal species (10) and have been implicated in causing human cancers. Nitrosamines rarely induce colonic cancer in experimental animals. However, *N*-nitrosamides such as *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10), caused colo-rectal carcinoma in rats when applied intrarectally.

Carcinogens require metabolic activation to exert their carcinogenic effect (21). Carcinogens, implicated in colon carcinoma, could be activated enzymatically in tissues other than the colon and reach the

target tissue via the blood circulation (22); (b) the intestinal lumen by deconjugation of metabolites by the microflora (23, 24); and (c) the intestinal mucosa by various enzymes e.g. the mixed-function oxidases (24). We have previously shown that both human and rat colonic mucosa in culture can activate procarcinogens into metabolites that bind to DNA; explants of human colon can metabolize DMN, 1,2-dimethylhydrazine and benzo[*a*]pyrene (BP) (13, 25). This observation suggests the importance of the third pathway described above.

A 50-fold inter-individual variation was found in the binding of DMN to human colon DNA, lower than the 100-fold variation observed in the binding levels of BP to DNA in cultured human colon (26) and the 75-fold variation in the binding levels of BP to DNA in cultured human bronchus (27). Several factors for this variation were considered. The intra-individual variation due to the methodology was minimal, i.e., coefficient of variation 0.1 (13). The viability of the tissue as monitored by high-resolution microscopy was good in all the reported cases; however, changes in cellular physiology could, in part, account for some of the observed differences. There is a positive correlation between the level of radioactivity associated with DNA and  $\text{CO}_2$ -formation. Alkylation took place at both the O-6 and N-7 position giving a ratio of 0.5. However, this radioactivity only accounted for a small part of the total radioactivity. Treatment of the material in the initial peak with strong acid, released about 40% of the radioactivity in form of methanol, indicating that the major alkylation site could either be the phosphate groups or the oxygens in thymidine and/or cytosine. This finding however requires further investigation. Incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$ - $\text{CO}_2$  in the purine ring of the nucleic acids by *de novo* synthesis could also account for some of the radioactivity associated with DNA (13). Human liver slices (28) and human bronchus (29, 30) are also able to metabolize DMN into  $\text{CO}_2$  and alkylating species which reacted with DNA. DMN has been shown mainly to alkylate the O-6 and N-7 positions of guanine in DNA (31); the ratio of methylation of O-6 to N-7 being nearly 1.1 in cultured human bronchus (30), while a lower ratio was found in animal experiments (32).

III. METHYLATION OF HUMAN COLONIC DNA BY [ $^{14}\text{C}$ ]DMN.

Base	dpm <sup>a</sup>
O <sup>6</sup> -MeGua	20 (2)
N <sup>7</sup> -MeGua	38 (13)
Guanine	57 (4)
Initial peak	1175 (84)

Numbers in parentheses, percentage of the total dpm added to the column.

The ability of the colon to metabolize the different *N*-nitrosamines varies among individuals. While colon from all investigated cases could metabolize DMN, only two cases could metabolize DEN into metabolites which reacted with DNA. Since the [ $^{14}\text{C}$ ]-atom is located at the two-position of the ethyl group the alkylating moiety can be deduced as being an ethyl group. NPy was also metabolized by colon from all the cases. Binding of both  $^3\text{H}$ - and [ $^{14}\text{C}$ ]NPy suggests that an adduct(s) is formed between a metabolite of NPy and DNA. Opening of the ring in NPy indicated by  $\text{CO}_2$ -formation suggests that several possibilities for alkylating species exist. Lack of correlation between alkylation of DNA by NPy and  $\text{CO}_2$ -formation could also implicate a more complex pattern of metabolism. It has been suggested that two of the reaction-products between NPy and nucleic acids are 7-(2-carboxy)ethylguanine and/or 7-methylguanine (33). However, a recent observation indicates that the alkylation species could be 3-formyl-1-propanediazohydroxide (34). The molecular structure of the DNA adduct in human colon is under investigation. Formation of  $^{14}\text{C}$ - $\text{CO}_2$  *in vivo* by rats injected with either 2,5-[ $^{14}\text{C}$ ]NPy or 3,4-[ $^{14}\text{C}$ ]NPy shows that ring oxidation occurs at both two and three positions (33). DNP had a high binding level to protein, while binding to DNA was only observed in one case. This observation of a high level of protein binding is similar to our results from cultured human bronchus (16).

*N*-nitrosamines may reach the colonic mucosal epithelial cells by several routes, where they could be metabolically activated. DMN has been detected in the blood of people ingesting both spinach and bacon; spinach is recognized as a rich source of nitrate/nitrite (35). *N*-nitrosamines have also been detected in the feces of human subjects, whose diet did not contain any detectable *N*-nitrosamines indicating that the compounds were formed *in situ* (36).

The etiology of human colonic cancer is a complex problem. No exogenous chemical compounds have been so far proven to cause this carcinoma in the human. Our observations, that human colonic mucosa can activate several types of procarcinogens (e.g. BP, 7,12-dimethylbenz[*a*]anthracene, 1,2-dimeth-

ylhydrazine and aliphatic *N*-nitrosamines) into forms that bind to DNA, suggests that the colon should be added to the list of organs which are likely to be susceptible to the carcinogenic action of these compounds.

**Summary.** Cultured human colon mucosa was found to metabolize both acyclic and cyclic *N*-nitrosamines as measured by  $^{14}\text{C}$ - $\text{CO}_2$  formation and reaction of the activated moieties with cellular macromolecules. Dimethylnitrosamine and *N*-nitrosopyrrolidine were metabolized by explants from all patients studied. A positive correlation between binding of dimethylnitrosamine to DNA and  $\text{CO}_2$ -formation was observed. DMN alkylated DNA in both O-6 and N-7 position of guanine. However, most of the radioactivity was associated with an acid labile compound. High binding levels of *N,N'*-dinitrosopiperazine to protein without concomitant binding to DNA were detected. Inter-individual variation in both binding level to DNA and ability to metabolize the different *N*-nitrosamines was observed.

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## Effects of Thyroxine, Epinephrine and Cold Exposure on Lipolysis in Genetically Obese (ob/ob) Mice<sup>1</sup> (40295)

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Mayer and Barnett (1) observed that genetically obese (ob/ob) mice were unable to withstand exposure to a cold environment and that the administration of thyroid hormone before cold exposure slightly prolonged their survival. More recent studies provided evidence that ob/ob mice were hypothyroid (2, 3) and that thyroid hormone administration corrected the observed hypothermia during cold exposure (4). Experimentally produced hypothyroidism in rats prevents normal epinephrine-stimulated lipolysis (5), and *in vitro* studies using adipose tissue from ob/ob mice have demonstrated a similar reduction in epinephrine-stimulated free fatty acid (FFA) release (6-8). These observations support the hypothesis that hypothyroidism in ob/ob mice results in defective lipolysis, thus limiting FFA as a substrate for thermogenesis during cold exposure. However, *in vivo* studies in ob/ob mice at ambient temperature failed to show defective lipolysis either in response to catecholamines (9) or during fasting (10).

In order to examine this apparent discrepancy between the *in vivo* and *in vitro* data in the literature and to study the metabolic effects of cold stress in ob/ob mice more precisely, an experiment was designed to investigate the hormonal influences on lipolysis and the relevant parameters of carbohydrate metabolism during cold exposure in these animals. Specifically, this study measured the effects of pharmacological doses of thyroxine ( $T_4$ ) on both *in vivo* and *in vitro* FFA release in cold-exposed ob/ob mice in comparison to the effects in non-obese mice. In addition, the effect of  $T_4$  treatment on epinephrine-stimulated FFA release from adipocytes was assessed.

**Materials and methods.** Male weanling mice of the obese strain, C57BL/6J-ob were purchased from Jackson Laboratories, Bar Harbor, ME. The obese (genotype, ob/ob) and non-obese (genotypes, +/ob and +/+) mice were fed an experimental diet containing 20% casein, 32% glucose, 32% sucrose, 10% corn oil, 5% salts (11), 0.5% vitamin mix (12) and 0.2% choline chloride. At 18 weeks of age and prior to  $T_4$  treatment and cold exposure, nonfasting blood samples were taken from the retro-orbital sinus in heparinized tubes for basal glucose and insulin determinations. At 24 hr before cold exposure, half of the obese and nonobese mice were injected ip with 100  $\mu$ g L-thyroxine (Sigma Chemical Co., St. Louis, MO) in 0.25 ml of 0.9% NaCl adjusted to pH 12 with NaOH, and the remaining half were injected with alkaline NaCl alone. These injections were repeated immediately before cold exposure. After 90 min at 4°, animals were killed by decapitation and blood collected in heparinized tubes for determinations of plasma glucose (13), insulin (14), and FFA (15).

*In vitro* lipolysis was measured in preparations of adipocytes isolated from 1 g portions of epididymal adipose tissue by the method of Rodbell (16). The washed fat cells were suspended in 9 ml of Krebs-Ringer bicarbonate buffer containing 3% fatty acid-free albumin. Three ml of this suspension was used to determine DNA (17). For determination of FFA release, 0.9 ml samples of fat cell suspension were incubated in duplicate vials for two hours at 37° in 2.1 ml Krebs-Ringer bicarbonate buffer containing 3% fatty acid-free albumin with and without  $1.1 \times 10^{-5}$  M epinephrine (Fisher Scientific Co., Fairlawn, NJ). At the end of the 2-hr incubation period, these test samples containing fat cells and incubation medium were cooled to 4°, homogenized and extracted for lipid (18). Zero time samples were prepared in duplicate by adding 0.9 ml portions of fat cell

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suspension to 2.1 ml of buffer at 4°. They were homogenized without incubation and the lipid was immediately extracted (18). The FFA content of lipid extracts from zero time and test samples was determined (15) and the total FFA content of the homogenates of fat cells plus incubation medium was calculated. The FFA in zero time samples was measured to provide an index of intracellular levels of FFA after cold exposure, as well as for calculation of FFA release from triacylglycerol during the incubation period, since the FFA content of the zero time samples was subtracted from that of the incubated test samples. FFA release was expressed as  $\mu\text{eq FFA}/\mu\text{g DNA}/\text{hr}$ . Statistical comparisons were made by Student's *t* test (19).

**Results.** The effects of  $T_4$  treatment on plasma glucose, insulin and FFA after cold exposure for 90 min are presented in Table I.  $T_4$  treatment had no statistically significant effect on any of these parameters in either obese or nonobese mice. However, there was a tendency toward higher plasma FFA values in  $T_4$ -treated mice, particularly for the obese.

Under non-fasting conditions at ambient temperature, obese mice had plasma glucose values of  $228 \pm 20$  (mean  $\pm$  SE) mg/dl and insulin values of  $96 \pm 8$   $\mu\text{U}/\text{ml}$ , while non-obese mice had glucose values of  $187 \pm 19$  mg/dl and insulin values of  $24 \pm 1$   $\mu\text{U}/\text{ml}$ . In comparison to these basal values, the elevated glucose and depressed insulin values shown in Table I indicate the response to the stress of cold exposure in both obese and nonobese mice. The higher plasma glucose and insulin values of the obese in comparison to nonobese mice under basal conditions are characteristic of this genotype. These same differences were observed in cold-exposed

obese and nonobese mice.

The intracellular concentrations of FFA in isolated adipocytes are also shown in Table I. Adipocytes from obese mice treated with  $T_4$  had a significantly higher concentration of FFA than those from untreated obese mice. In contrast, fat cells from nonobese mice had similar FFA concentrations regardless of treatment, and these were not significantly different from the mean value for untreated obese mice. This elevated zero time FFA concentration only in  $T_4$ -treated obese mice suggests an increased *in vivo* lipolytic response to  $T_4$  in these animals.

Figure 1 illustrates the results of the meas-

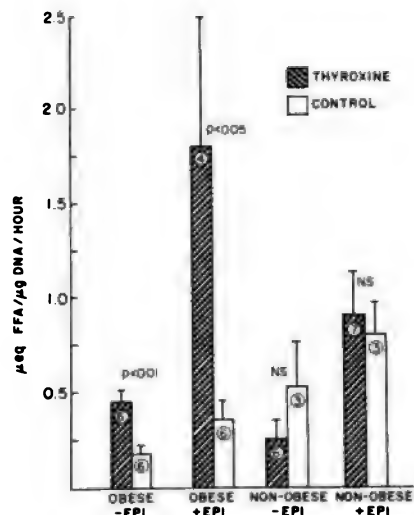


FIG. 1. Effect of  $T_4$  treatment *in vivo* on production of FFA by hydrolysis in fat cells isolated from epididymal adipose tissue of cold-exposed mice and incubated with (+) and without (-)  $1.1 \times 10^{-5}$  M epinephrine (EPI). Number of animals are indicated on each column, bars represent SEM, and P values compare differences between paired column means.

TABLE I. EFFECT OF THYROXINE TREATMENT ON OBESE AND NONOBESE MICE EXPOSED TO THE COLD (4°) FOR 90 MIN.

Group	Treatment	Plasma glucose (mg/100 ml)	Plasma insulin ( $\mu\text{U}/\text{ml}$ )	Plasma FFA ( $\mu\text{eq}/\text{liter}$ )	Adipocyte FFA ( $\mu\text{eq}/\mu\text{g DNA}$ )
Obese	Saline	$379 \pm 54$ (6)*	$24 \pm 5$ (6)	$722 \pm 142$ (6)	$0.25 \pm 0.08$ (6)
	Thyroxine	$375 \pm 64$ (6)	$23 \pm 3$ (6)	$855 \pm 208$ (6)	$1.33 \pm 0.43$ (6)
		NS	NS	NS	$P < 0.05$
Non-obese	Saline	$256 \pm 58$ (6)	$17 \pm 1$ (6)	$697 \pm 75$ (4)	$0.37 \pm 0.09$ (3)
	Thyroxine	$237 \pm 11$ (7)	$18 \pm 1$ (7)	$716 \pm 65$ (5)	$0.41 \pm 0.05$ (7)
		NS	NS	NS	NS

\* Values are mean  $\pm$  SE. Number of mice sampled in parentheses.

urements of *in vitro* lipolysis after cold exposure for 90 min. Lipolysis in isolated adipocytes is presented as  $\mu\text{eq}$  FFA released per  $\mu\text{g}$  DNA per hr of incubation in order to express the results in terms related to cell number rather than cell mass. The data from the untreated control mice show that adipocytes from cold-exposed obese mice had reduced lipolytic activity in comparison to those from nonobese mice. The addition of epinephrine to the incubation medium of fat cells from both obese and nonobese control mice increased FFA release, but the values were not significantly different from those under non-stimulated conditions. The epinephrine-stimulated release of FFA from the fat cells of untreated obese mice remained significantly ( $P < 0.05$ ) lower than the release from fat cells of untreated nonobese mice.

$T_4$  treatment of obese mice before cold exposure had a striking effect on *in vitro* lipolysis in contrast to the small but not significant effect in nonobese mice (Fig. 1). Adipocytes from  $T_4$ -treated obese mice released significantly more FFA both in the presence ( $P < 0.05$ ) and in the absence ( $P < 0.01$ ) of epinephrine in comparison to adipocytes from corresponding untreated obese mice. The response to epinephrine of adipocytes from  $T_4$ -treated obese mice was more than three times greater than that of fat cells from untreated control obese mice. However, adipocytes from nonobese mice showed no increase in FFA release when treated with  $T_4$  prior to cold exposure, although there was a significant ( $P < 0.05$ ) rise in FFA release in response to epinephrine in  $T_4$ -treated non-obese mice.

**Discussion.** From the data presented it is apparent that the failure of ob/ob mice to survive during cold exposure was not attributable to insufficient circulating FFA since plasma values in obese mice were comparable to those in nonobese mice after cold exposure for 90 min at  $4^\circ$ . Other studies in this laboratory (20) showed that a more prolonged cold exposure (up to 4 hr) also resulted in similar plasma FFA values in obese and non-obese mice. The plasma FFA values obtained in the present experiment were similar to those found by Abraham *et al.* (9) for obese and nonobese mice after norepinephrine administration and after a 24-hr fast. Their

study and our study during cold stress showed normal *in vivo* lipolysis for ob/ob mice without thyroid hormone treatment, which is known to alleviate the hypothermia in ob/ob mice. Thyroid hormone also did not significantly alter FFA values *in vivo*.

The *in vitro* results indicated an inhibition of basal and epinephrine-stimulated lipolysis in adipocytes from ob/ob mice after cold stress, a condition in which lipolysis should be maximally stimulated. This was similar to the inhibition of FFA release from adipose tissue of ob/ob mice found at ambient temperature by other investigators (6–8), although that Marshall and Engel (6) did not observe inhibition under basal conditions (without epinephrine). In addition, Herberg *et al.* (7) reported increased release of FFA from epididymal adipose tissue under both basal and epinephrine-stimulated conditions. However, these latter investigators pre-incubated adipose tissue in Krebs-Ringer bicarbonate buffer with albumin and glucose (not only before measuring lipolysis but also before insulin was “washed out” by this preincubation and no longer exerted its known inhibitory effect on lipolysis (22)). Otto *et al.* (8) found elevated lipolysis as measured by glycerol release from adipose tissue of ob/ob mice under basal conditions, but reduced sensitivity to epinephrine and thyroid hormone administration. Although FFA release is a parallel glycerol release during lipolysis, it may not occur under these conditions. Differing from ours, in which adipose tissue from ob/ob mice in contrast to adipocytes were incubated in the presence of glucose.

The coexistence of our *in vivo* results showing similar plasma FFA after cold exposure in both  $T_4$ -treated and untreated obese and nonobese mice and the *in vitro* results showing variable FFA release from fat cells is possible for several reasons. First, when fat cells from untreated obese mice release FFA on a cell number basis, the increased number of fat cells in these obese mice could be sufficient to maintain plasma FFA at similar concentrations to those in nonobese mice. Also, *in vitro* conditions are not necessarily analogous to those *in vivo*. For example, it is possible that a more rapid turnover of circulating FFA or an inhibition of FFA release in the blood occurs *in vivo*. FFA determinations

also made on homogenates of fat cells in incubation medium and, therefore, reported fatty acid release from triacylglycerol not necessarily release from fat cells. Han *et al.* (24) have shown that under certain circumstances intracellular FFA concentrations increased without increasing FFA release from the cell. Since the intracellular FFA concentration in the  $T_4$ -treated obese mice was significantly higher than in any other group, as determined from the zero time samples, it is possible that all of the FFA released during lipolysis *in vivo* were not re-enters into the circulation.

The significant increase in FFA release from lipid stores in adipocytes in response to cold exposure in both nonobese and obese mice treated with  $T_4$  is in agreement with observations in other rodents in which  $T_4$  potentiated the action of epinephrine (25) and in the lipolytic response of adipocytes *in vitro* as affected by the *in vivo* thyroid status of the animal (5, 26). However, the accentuation of epinephrine-stimulated lipolysis in mice treated with  $T_4$  as compared to the effect in nonobese mice suggests an increased sensitivity to epinephrine in obese mice once the hypothyroid status is corrected. Although this study did not directly test the hypothesis that decreased thermogenesis in obese mice during cold exposure was a result of decreased FFA availability, the evidence of reduced lipolysis by adipocytes was reversed by  $T_4$  treatment supports the hypothesis. Although circulating FFA concentrations were not significantly affected by treatment, it is possible that  $T_4$  potentiated the rate of FFA release from adipose tissue *in vivo* as well. A similarly increased FFA uptake and oxidation could allow increased thermogenesis, while maintaining plasma FFA concentrations constant.

**Summary.** Treatment of ob/ob mice with  $T_4$  prior to cold exposure did not alter plasma concentrations of glucose, insulin and during cold exposure although ob/ob mice remained hyperglycemic and hyperinsulinemic when compared to nonobese mice. Content of and FFA release from adipocytes were significantly elevated in treated obese mice after cold stress as compared to untreated obese mice.  $T_4$  treatment also produced a marked increase in

epinephrine-stimulated FFA release from fat cells of obese mice *in vitro*.

These results indicate that correction of the hypothyroid status of ob/ob mice with pharmacological doses of  $T_4$  improved the *in vitro* lipolytic response of fat cells, but did not alter the circulating concentrations of important energy sources for thermogenesis *in vivo*.

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## Apomorphine-Induced Inhibition of Episodic LH Release in Ovariectomized Rats with Complete Hypothalamic Deafferentation<sup>1</sup> (40296)

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Laboratory recently reported that apomorphine, a drug that stimulates dopamine release, caused a transient (50–60 min) but inhibition of the episodic pattern of release normally observed in ovariectomized rats (1, 2). This effect is mediated by stimulation of dopamine receptors since pindolol and butaclamol, agents which block dopamine receptors, prevent the inhibitory effect. The present study was designed to determine if this inhibition is mediated by an activation of dopamine receptors in the hypothalamic-pituitary unit (3, 4), or if it is in some other region of the brain with a significant dopaminergic input to the neostriatum (3). Therefore, the effects of apomorphine on episodic LH release were determined in ovariectomized rats previously subjected to complete hypothalamic deafferentation in order to isolate the basal hypothalamus (MBH)-pituitary from the rest of the brain.

**Materials and methods.** Adult female Sprague-Dawley rats (Simonsen Laboratories, CA) weighing 260–280 g were placed on a lighting schedule of 14 hr light/10 hr darkness (light on 0500–1900 hr) and fed lab chow and water *ad libitum*. Daily vaginal smears were taken and only those rats with two or more consecutive 4-day estrous cycles were used for experimentation. Deafferentation of the MBH was performed with a small double-edged Halasz knife (5) of bayonet shape (dimensions: 2.0 mm, radius 1.6 mm). Under sodium pentobarbital anesthesia (35 mg/kg), the animal's head was placed in a stereotaxic instrument with the ear bars 2.4 mm above the level of the tooth bar. After drilling

a hole in the skull, the knife was lowered through the superior sagittal sinus to the base of the skull 8.3 mm anterior to the interaural line. The knife was first rotated to the right 90°, and then 180° to the left (to maximize the probability for completeness of the anterior section of the cut). The blade was next stereotactically moved 3 mm posteriorly, and then rotated 180° to the right. It was then moved anteriorly 3.3 mm (to assure completeness). Finally, the blade was rotated 90° toward the starting position, and removed from the brain at the point of entry. Following deafferentation, vaginal smears were taken for 3 to 6 weeks after which time only those rats having shown either constant vaginal estrous or diestrous smear patterns for three weeks or more were ovariectomized.

Six weeks following ovariectomy a polyethylene cannula was inserted into the external jugular vein and used for collecting blood samples the following day. An additional cannula was placed subcutaneously in the animal's back for later drug administration. The next day, after an iv injection of 200 units heparin, unanesthetized, unrestrained animals were bled continuously through a piece of flexible tubing, one end of which was connected to the animal's cannula and the other end through a peristaltic pump to a microliter syringe kept on ice for the collection of blood samples. Fifty or 100 µl whole blood were collected every 5 or 10 min, respectively, and added directly to assay tubes (kept in an ice bath) containing 400 or 450 µl of phosphate buffered saline with 0.1% gelatin. After collecting blood samples for a 1½- to 2 hr-control period, animals were injected with apomorphine hydrochloride (a selective stimulator of dopamine receptors (6, 7), Merck Chem., Rahway, NJ, 1.5 mg/kg in saline) through the indwelling sc cannula. Bleeding was then continued for an additional 1 to 1½-hr period. Whole blood sam-

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ples were analyzed for LH by a slight modification (8) of the ovine-ovine rat LH double antibody radioimmunoassay of Niswender *et al.* (9). LH values (ng/ml whole blood) are expressed in terms of the NIAMDD Rat LH-RP-1 preparation which has a biological potency equivalent to  $0.03 \times \text{NIH-LH-S1}$ .

Following experimentation, rats were perfused with 10% formalin plus 1% calcium chloride. The extent of hypothalamic deafferentation was determined both by visual examination of the cut at the base of the brain as well as by close histological examination after sectioning brains at  $50 \mu\text{m}$  in the transverse plane and staining with Nissls stain using basic fuchsin.

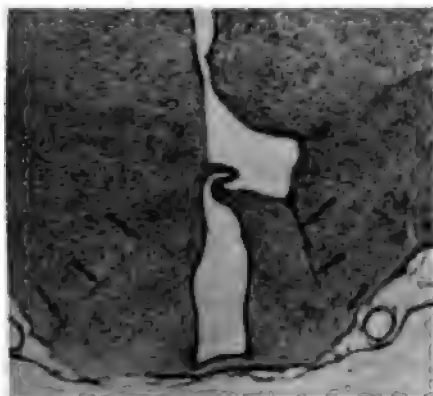
**Results.** Forty-five of 53 animals (85%) showed persistently leucocytic (constant diestrous) vaginal smear patterns for at least 3 weeks following hypothalamic surgery. The remaining eight rats (15%) exhibited persistent vaginal cornification (constant estrous) during this same period of time. No hypothalamic necrosis was observed in the great majority of animals subjected to deafferentation and later used for experimentation. The necrosis that was seen in a few rats involved only the extreme rostral or caudal sections of the deafferented tissue and never involved the arcuate nucleus-median eminence region. The pituitary glands of all experimental animals were not damaged by the knife. Additionally, no apparent histological differences with regard to the extent of deafferentation were discernible between constant estrous and constant diestrous animals (see Fig. 1). The deafferented tissue included all of the arcuate nucleus and median eminence, much of the ventromedial nucleus, and variable amounts of the dorsomedial nucleus. The posterior part of the suprachiasmatic nucleus was included within one side of the hypothalamic island in 2 of 8 constant estrous and 3 of 12 constant diestrous rats.

Twelve of the 45 rats displaying a persistently leucocytic smear pattern following hypothalamic deafferentation were randomly selected for bleeding 6 weeks after ovariectomy. In all 12 animals pulsatile LH release was absent and LH levels were very low ( $<28$  to  $<110 \text{ ng/ml}$ ). The rat in constant diestrous, depicted in Fig. 1, had  $<28 \text{ ng LH/ml}$  whole blood during a 3 hr bleeding period. Of the

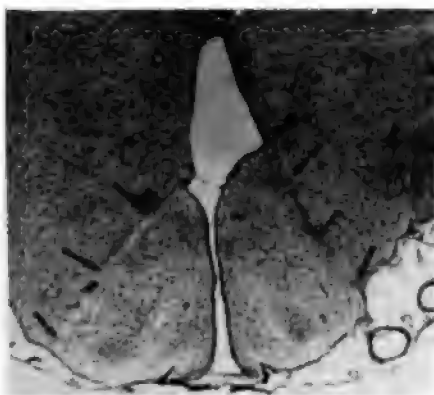
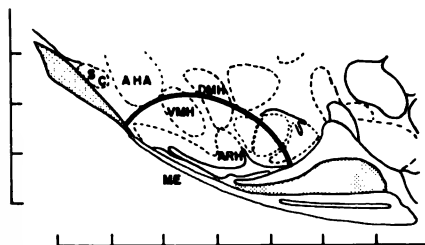
eight completely deafferented, constant estrous animals bled 6 weeks after ovariectomy, five exhibited pulsatile LH release during a  $1\frac{1}{2}$ - to 2 hr-control period of bleeding (Fig. 2), though at somewhat reduced levels when compared with pulsatile LH release normally seen in ovariectomized rats. In the remaining three rats, problems occurred during the bleeding procedure in one, while the other two animals displayed either nonepisodic, low blood LH levels or only one LH pulse in the control period.

Apomorphine caused a stereotyped gnawing behavior pattern in ovariectomized rats with complete hypothalamic deafferentation, much as it does in intact or ovariectomized animals not subjected to hypothalamic surgery (1, 2, 6). This agent was administered to eight rats with complete hypothalamic deafferentation which previously had shown constant vaginal estrous smear patterns before ovariectomy. In the five rats having well defined episodic LH release patterns during the control period, apomorphine caused an inhibition (four rats) or reduction (one rat) of pulsatile LH secretion lasting at least 40–90 min. Three examples are given in Fig. 2. The extent of the cut in the middle animal represented in Fig. 2 is shown in the top of Fig. 1. The response to apomorphine could not be determined in the remaining 3 rats because of the reasons cited above.

**Discussion.** This study demonstrates that apomorphine, a specific dopamine receptor stimulating agent (6, 7), can exert an inhibitory effect on episodic LH release in ovariectomized rats previously subjected to complete hypothalamic deafferentation. We have previously observed this inhibition in ovariectomized animals not subjected to complete hypothalamic deafferentation (1, 2) and have shown that the sc injection of saline (1) or distilled water (2) into ovariectomized rats had no effect on episodic LH release. Furthermore, the sc injection of apomorphine into animals with hypothalamic deafferentation was accomplished through the use of an indwelling sc cannula connected to a sufficient length of flexible tubing to extend out of the animal's cage. Thus, the animals were unaware of any injection procedure. It appears from these and our previous data that the inhibition of episodic LH release caused



## Constant estrus



## Constant diestrus

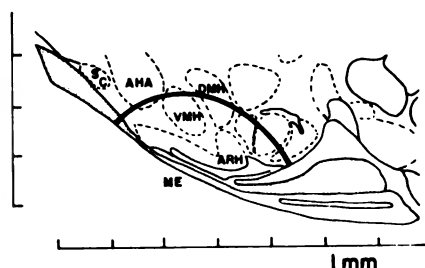


FIG. 1. Representative sagittal reconstructions indicating the extent of complete hypothalamic deafferentation in rats subsequently showing constant estrous or constant diestrous vaginal smear patterns. Actual brain cross sections for each of these animals are shown to the left. The arrows indicate the location of the knife cut.

by apomorphine is a result of activation of dopamine receptors within the medial basal hypothalamus (MBH) and/or pituitary gland, and not outside this region.

The postsynaptic dopamine receptors responsible for inhibition of episodic LH release are probably associated with neurons innervated either by dopaminergic neurons originating in the arcuate nucleus or within the substantia nigra, and both these areas send axonal projections to the median eminence (10-13). In this regard, the median eminence contains high concentrations of LHRH (14, 15), apparently within the terminals of LHRH neurons. It is possible that activation of dopamine receptors on these LHRH neurons may result in an inhibition of LHRH release. A hypothalamic site of action for apomorphine is suggested by the

evidence that portal vein infusion of dopamine had no effect of LH release (16), while the *in vitro* pituitary secretion of LH was inhibited by dopamine only when the median eminence was included in the incubation (17). Alternatively, a pituitary site of action cannot be ruled out since dopamine receptors are present there (4).

It should be emphasized that the inhibition of episodic LH release by apomorphine could only be tested in those few hypothalamic-deafferented animals showing a constant vaginal estrous smear pattern, since only in these rats was episodic LH release present after ovariectomy. The vast majority of deafferented rats (85%) exhibited a constant vaginal diestrous smear pattern and in this type animal LH levels were very low and nonpulsatile after ovariectomy. Blake and Sawyer (18)

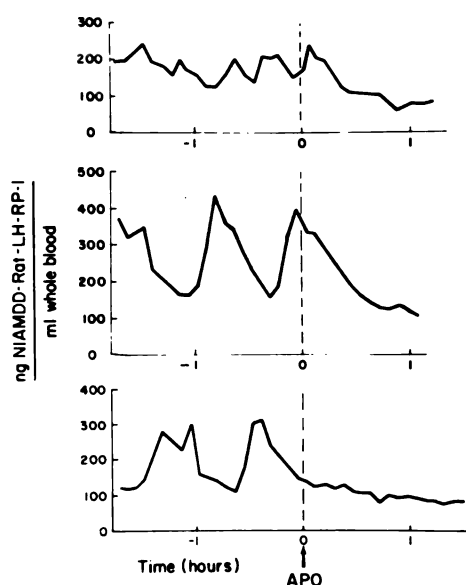


FIG. 2. Three examples of the effect of apomorphine (APO; 1.5 mg/kg sc) on episodic LH release in ovariectomized rats with complete deafferentation of the medial basal hypothalamus.

indicated that 5 of 11 animals subjected to complete deafferentation of the MBH had constant vaginal estrous smear patterns and episodic LH release after ovariectomy. These authors suggested on the basis of these animals that pulsatile LH secretion may possibly be inherent to the MBH-pituitary unit. In only a small percentage of the rats in the present report was the deafferented hypothalamic tissue capable of maintaining episodic LH secretion. In agreement with Blake and Sawyer (18), complete MBH deafferentation also had produced constant vaginal cornification in these rats. Inclusion of the supra-chiasmatic nucleus within the deafferented region has been suggested to account for the persistence of LH secretion and this constant vaginal estrous smear pattern (19). In the present study the supra-chiasmatic nucleus was anterior to, or destroyed by the knife cut in the large majority of rats in both groups. Moreover, even when a portion of this nucleus was included within the deafferented hypothalamic tissue in a few constant diestrous rats, very low, nonepisodic blood LH levels still resulted. Thus, the reason why some rats should continue to show episodic LH release while others do not, when the

extent of hypothalamic deafferentation appears similar in both groups, is not present. The absence of pulsatile LH secretion following MBH deafferentation due to severing the axons of LHRH whose cell bodies lie outside the MBH interrupting fibers stimulating LH synthesis and/or release. Complete deafferentation results in a large decrease in LH content in the rat MBH (20, 21). Moreover, norepinephrine has been suggested to play an excitatory role in the regulation of LH secretion (1, 22-25) and the  $\alpha$ -norepinephrine in the MBH is depleted by deafferentation (26). Nevertheless, afferent input to the MBH seems to be required in the great majority of rats to sustain episodic LH secretion. Moreover, dopamine receptors within the MBH-pituitary seem responsible for mediating the inhibitory effect of apomorphine on pulsatile LH secretion.

**Summary.** Complete neural deafferentation of the MBH in 53 rats resulted in a constant vaginal diestrous smear pattern in 85% of the rats, and in this type animal low blood LH levels and absence of episodic LH release followed ovariectomy. The remaining 15% had a constant vaginal estrous smear pattern, and most demonstrated episodic LH secretion following ovariectomy. Thus, afferent input to the MBH seems to be required in most rats to sustain episodic LH secretion. Administration of apomorphine, a dopamine receptor stimulator, to ovariectomized rats in constant estrus before ovariectomy, resulted in inhibition of pulsatile LH secretion, suggesting that this apomorphine-induced inhibition is a result of activation of dopamine receptors within, rather than outside, the MBH-pituitary unit.

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# The Effect of Leukocyte Hydrolases on Bacteria. XI. Lysis by Leukocyte Extract by Myeloperoxidase of a *Staphylococcus aureus* Mutant Which is Deficient in Ribitol Teichoic Acid, and the Inhibition of Bacteriolysis by Lipoteichoic Acid<sup>1</sup> (40297)

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In previous publications, we have shown that *Staph. aureus*, which had been harvested from the logarithmic phase of growth, was readily lysed by human leukocyte extracts (ENZ) and by myeloperoxidase (MPO). On the other hand, bacteria obtained from the stationary phase of growth were highly resistant to degradation by these agents (1-8). It was further demonstrated that the lysis of the bacteria by the leukocyte factors was probably caused by the activation of autolytic systems and not by the direct effect of lysosomal hydrolases on the bacterial walls (8). It was suggested that one of the reasons for the resistance to degradation of the stationary phase bacteria was not due to the lack of autolytic enzymes in the old cells, but to the much thicker cell walls of such cells (7, 8).

It is well established that Gram positive bacteria possess teichoic acid (TA) as an integral part of the cell wall and a membrane-associated lipoteichoic acid (LTA) (9). Since TA was claimed to deter the interaction of lysozyme with the peptidoglycan, thus conferring resistance to bacteriolysis (10), and since LTA has been implicated in the inhibition of autolytic enzymes in bacteria (11, 12), it was of interest to test the effect of TA and LTA on bacteriolysis induced by leukocyte factors. The data presented show that a mutant of *Staph. aureus*, which completely lacks ribitol TA but nevertheless possesses membrane-associated LTA, is much more susceptible to lysis by ENZ and by MPO than the parent strain. It will also be shown that while LTA strongly inhibited the lysis of *Staph. aureus* by ENZ and by MPO, TA was

not inhibitory.

**Materials and methods.** *Microorganisms.* The following *Staph. aureus* strains were employed: The parent strain SH (Str<sup>r</sup>) mutants 52A5 and 52A2. The mutant 52A5 lacks ribitol teichoic acid in the cell wall, no ribitol phosphate polymer was detected in any other cell fraction or in the supernatant. The lack of ribitol teichoic acid in the cell wall is caused presumably by some defect in the membrane or in some unknown factor required in the polymerization or at some step of the teichoic acid to murein. The mutant 52A2 lacks *N*-acetylglucosaminidase and cell wall ribitol teichoic acid. All the mutants are also known to be deficient in penicillinase (for details see reference 13). These mutants were kindly supplied by Dr. D. M. Brown from the Department of Biophysics, Weizmann Institute, Rehovoth, Israel. In addition we have employed *Staph. aureus* strain Cowan I which is known to lack protein A. The bacterial strains were grown in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI) or BHI which contained 0.5  $\mu$ Ci/ml of radioactively labeled [<sup>14</sup>C]-D-glucose, specifically 150-250 mCi/mmol (New England Nuclear, Boston, MA) as described (2). All bacterial cells were harvested either from the logarithmic phase of growth (after 3 hr of incubation, OD = 280 Klett units at 540 m $\mu$ , Klett Summerson colorimeter) or from the stationary phase of growth (after 18 hr of incubation, OD = 620 Klett units at 540 m $\mu$ ). Cells were washed several times in saline and resuspended in distilled water.

**Lipoteichoic acid (LTA).** Lipoteichoic acid (LTA) was isolated from *Strep. mitis* (ATCC 1895/74), group A streptococcus (C203S) and from *Staph. aureus* (S 52A2 and cowan I) by phenol (Mallinckrodt, St. Louis, MO) or by lysozyme

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ical Co., St. Louis, MO) (14). The bacterial extracts were dialyzed with six changes distilled water and were then lyophilized, preparation did not contain any traces enol. Teichoic acid (TA) was isolated *Staph. aureus* SH by TCA (BDH, Eng- according to a method described by mo and Slade (16).

acylation of LTA. was performed as de- d by Knox and Wicken (9).

opolysaccharide (LPS). LPS from *E. coli* 38 was purchased from Difco Labora- (Detroit, MI). The LPS was dissolved ine to the desired concentration.

duction of anti-LTA serum. Antibodies st LTA were prepared by immunizing s either with *Strep. mutans* SE 1895/74, *Staph. aureus* Cowan I or with group A streptococci, according to a procedure bed in detail (14).

termination of LTA activity. LTA was nined quantitatively by its ability to ize human RBC to agglutination in the ice of a standard anti-LTA serum as bed (16).

teriolysis. The lysis of staphylococci was med as described in detail (2). Briefly, beled bacterial suspensions containing  $10^3$  cpm/100 Klett units per ml were ated for 18 hr at 37° in 0.1 M acetate pH 5.0 either with freeze and thaw ts of human blood leukocytes contain- 500 µg/ml of protein or with purified peroxidase which was kindly supplied r. I. Olsson from the Department of al Medicine University of Lund, Swe-

den, as well as with nuclear histone (Sigma Chemical Co., St. Louis, MO). MPO was used here as a cationic protein and not as a bactericidal agent which in collaboration with H<sub>2</sub>O<sub>2</sub> and halide is a strong bactericidal agent.

The degree of lysis was determined by measuring the percentage release of soluble radioactivity from the standard labeled bacterial suspension (2).

*The inhibition of bacteriolysis.* Radio-la- beled bacteria were incubated for 15 min at 37° with various amounts of LTA (phenol or lysozyme extract) with deacylated LTA (9) with TA or with LPS. Following incubation, the cells were lysed by leukocyte factors, and the degree of inhibition of lysis was deter- mined as described (2). The results were ex- pressed as the percentage of inhibition of the release of radioactivity from a standard sus- pension of [<sup>14</sup>C]labeled staphylococci.

*Results. The lysis of staphylococci by leu- kocyte factors.* Table I shows that when sta- tionary bacteria were employed, only strain 52A5 (which is deficient in TA) underwent massive lysis following treatment with human leukocyte extracts (ENZ). On the other hand, all the bacterial strains employed were equally susceptible to lysis when harvested from the logarithmic phase of growth (young cells). It is important to note that identical results were obtained when a purified prep- aration of myeloperoxidase (MPO) or histone were used instead of the leukocyte extracts (not shown). Table I also shows that strain 52A5 loses a somewhat higher percentage of radioactivity when incubated in buffer alone

TABLE I. THE LYSIS OF DIFFERENT STAPHYLOCOCCAL STRAINS BY LEUKOCYTE EXTRACTS.

Bacterial strain	Presence or absence of			% release of radioactivity from <sup>a</sup>			
	Protein A	TA	LTA <sup>b</sup>	logarithmic phase bacteria		stationary phase bacteria	
				Buffer	ENZ	Buffer	ENZ
I	—	+	+	21	80	18	38
	—	—	+	22	75	30	75
	—	± <sup>c</sup>	+	28	78	15	35
	+	+	+	25	92	10	30

diolabeled bacteria (100 Klett units/ml 540 µm) suspended in 0.1 M acetate buffer pH 5.0 were incubated for t 37° with 100 µg/ml of human leukocyte extracts (ENZ) and the soluble radioactivity was determined as ed in Materials and Methods. Similar results were obtained with MPO or histone. The data are the mean of periments.

A was extracted from the bacteria either with phenol or by lysozyme as described in Materials and Methods. is mutant lacks *N*-acetylglucosamine in its TA.



(spontaneous lysis) as compared with the other strains. Since all the bacterial strains employed were found to possess LTA (Table I), it is postulated that TA, but not the membrane-associated LTA, may play an important role in the protection of old bacteria against lysis by leukocyte factors.

*The inhibition by LTA of the lysis of staphylococci.* LTA was recently shown to be a potent inhibitor of autolytic enzymes in *Strep. faecalis* (11) and *Diplococcus pneumoniae* (12). Since we have recently postulated (8) that the lysis of *Staph. aureus* by leukocyte extracts and by membrane-damaging agents like MPO and Phospholipase A<sub>2</sub>, was due to the activation of autolytic enzymes, it was of interest to examine the possibility that LTA will also inhibit bacteriolysis induced by leukocyte factors and by MPO. Table II shows that when LTA (derived either from staphylococci or from streptococci) was added to staphylococci (SH and 52A5) in the presence of an inducer of lysis like ENZ or MPO, a strong inhibition of lysis occurred. It is also seen that H<sub>2</sub>O<sub>2</sub> did not modify either the lytic effect of MPO or the inhibitory effect of LTA on bacteriolysis induced by MPO. The Table also shows that neither deacylated LTA nor TA nor LPS had any inhibitory property. It is also shown that none of the inhibitors employed lysed the bacteria. In other experiments (not shown) we found that the lysis of staphylococci by ENZ could not be inhibited by cytoplasmic fractions or cell walls derived from group A streptococci, when used at similar concentrations.

*Discussion.* The data on the higher susceptibility to lysis of the TA-deficient mutant by leukocyte factors and by MPO and the inhibition of bacteriolysis by LTA, further contribute to the understanding of the possible role which may be played by TA and LTA in the biology of the staphylococci.

Since TA was claimed to deter the interaction of lysozyme with the peptidoglycan (10) it may be postulated that the lack of this wall component from the mutant 52A5 rendered the cell more susceptible to bacteriolysis. Since however, the lysis of *Staph. aureus* by leukocyte enzymes was found not to be lysozyme-dependent (3, 5) and since the TA-less mutant was not more susceptible to lysis by lysozyme than the parent strain (Table II),

TABLE II. THE EFFECT OF LTA, DEACYLATED LTA, TA AND LPS ON THE LYSIS OF STAPHYLOCOCCI LEUKOCYTE FACTORS.

Reaction mixture <sup>a</sup>	% Release of radioactivity after 18 hr from: <sup>b</sup>	
	Strain SH	Strain 52A5
Buffer alone	21	22
Lysozyme 100 µg	30	32
Leukocyte extracts 100 µg	80	75
MPO 100 µg	82	75
LTA <sup>c</sup> 250 µg	20	20
H <sub>2</sub> O <sub>2</sub> 0.3 µg	19	ND <sup>d</sup>
Leukocyte extracts + H <sub>2</sub> O <sub>2</sub> 0.3 µg	79	ND
Leukocyte extracts + LTA 150 µg	30	35
Leukocyte extracts + LTA 250 µg	25	28
Leukocyte extracts + LTA 500 µg	17	20
Leukocyte extracts + LTA 500 µg + H <sub>2</sub> O <sub>2</sub> 0.3 µg	14	ND
Leukocyte extracts + Deacylated LTA 150 µg	76	75
Leukocyte extracts + Deacylated LTA 250 µg	70	76
Leukocyte extracts + TA 250 µg	80	75
Leukocyte extracts + LPS 500 µg	78	72
MPO + H <sub>2</sub> O <sub>2</sub> 0.3 µg	72	ND
MPO + LTA 250 µg	23	25
MPO + LTA 500 µg	17	22
MPO + LTA 500 µg + H <sub>2</sub> O <sub>2</sub> 0.3 µg	15	ND

<sup>a</sup> The reaction mixtures were added to the label bacteria (logarithmic phase) in 0.1 M acetate buffer pH 5.0.

<sup>b</sup> Lysis was determined as the percentage of the solubilized radioactivity as described in Materials and methods. The data are the average of five experiments.

<sup>c</sup> All LTA preparations (see Materials and method) behaved similarly.

<sup>d</sup> ND—Not done.

one should seek other explanations for the higher susceptibility of the mutant to lysis by leukocyte factors.

It may be postulated that since old staphylococci (shown to be resistant to degradation) (Table I), possess much thicker cell wall (17) and since TA forms the bulk of the staphylococcus cell wall, it is possible that the lack of TA from the mutant renders the "thinner" cell wall of these microorganisms more susceptible to degradation by the autolytic enzymes, which are activated by the leukocyte factors (8). Thus TA may be essential for

ilization of the cell wall not only lysozyme but also against the autoly-

has been shown to be a potent inhibitory enzyme in a variety of bacteria (11, 12). The findings that exogenous LTA can inhibit the lysis, by ENZ (Table II) of staphylococci which endogenous LTA (Table I) is intrinsic to explain this phenomenon one may conclude that since leukocyte extracts, lysozyme and histone were shown to remove the LTA from bacterial cells (14), the lysis of the staphylococcus cells by leukocyte factors may involve, first the removal of endogenous LTA from the bacterial cells by the leukocyte factors, then the release from inhibition of the autolytic enzymes, and finally stimulation of the activity of the autolytic

enzymes shown in Table II lysis of staphylococci and other organisms known to produce  $H_2O_2$  is induced by MPO. These results are consistent, since neither KCN nor  $NaN_3$ , nor hemeprotein inhibitors, could inhibit bacteriolysis by MPO (unpublished observations). It thus points to the possibility that  $H_2O_2$  (a cationic substance) like other membrane-damaging agents, (e.g. LCP, histiophospholipase  $A_2$ , polymyxin B, colistimycin) may interact with the protoplast membrane and through perturbation, activate membrane-associated autolytic systems through the removal of LTA (14). This may also explain teleologically why PMN require large amounts of MPO.

The fact that  $H_2O_2$  did not modify the lytic effect of LTA, on the lysis of staphylococci by MPO (Table II), further supports the assumption that MPO in this system acts as a lytic protein.

Reasons for the use of acid buffers in the bacteriolytic system are based on our previous findings (5, 18) that optimal killing of staphylococci by leukocyte extracts and histone took place at pH 5.0, and only a slight effect was obtained at pH 7.0.

The interrelationships among TA, LTA, leukocyte systems and leukocyte factors in the degradation of microbial cell constituents in inflammatory sites merit further examination.

**Summary.** A *Staph. aureus* mutant (52A5) which is deficient in wall teichoic acid (TA) was found to be highly susceptible to lysis by leukocyte extracts (ENZ) and by myeloperoxidase (MPO) when harvested from the stationary phase of growth. On the other hand, a staphylococcus mutant, which is deficient in *N*-acetyl glucosamine in its TA (52A2), the parent strain SH and a protein A rich strain Cowen I, could be lysed by the leukocyte factors only when harvested from the logarithmic phase of growth.

The lysis of all the bacterial strains by ENZ or by MPO was strongly inhibited by lipoteichoic acid (LTA) derived either from staphylococci or from streptococci. On the other hand, deacylated LTA, TA, LPS, cytoplasmic or cell wall components derived from streptococci had no inhibitory effect on bacteriolysis. It is concluded that TA may be important in the protection of old bacterial cells against degradation by leukocyte factors, and that LTA may be involved in the control of autolytic enzymes in staphylococci. The role of MPO in bacteriolysis is also discussed.

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## Polybrominated Biphenyls in Chicken Eggs vs. Hatchability<sup>1</sup> (40298)

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In fall of 1973, polybrominated biphenyls (PBB) were accidentally introduced into poultry and livestock within Michigan. Millions of chickens and thousands of turkeys were destroyed to lessen the contamination of PBB into the food-chain (1). Fries (2) had reported that eggs from hens fed PBB averaged 21.5 ppm of hexabromobiphenyl (6-BB) plus heptabromobiphenyl (7-BB), and that 7 weeks after withdrawal of the PBB diet eggs contained 2.2 ppm of these compounds. Fat from these eggs contained 69.5 and 62.4 ppm of these compounds at the respective times of 9 weeks on diet and 7 weeks after their withdrawal. Residues were reported to be 1.5× the level of PBB after 4 weeks of feeding (3). This steady state effect was reported to occur as early as 10 days (4, 5). In data obtained from feeding PBB to turkeys, calculations (4) revealed a relation of PBB in eggs to be 1.3× the level in diet, similar to the 1.5 value calculated from the dose-response curve (5) and the 1.1 value by Cecil *et al.* (3).

Embryo mortality as a percent of fertile eggs averaged 6.2% and 3.9% for diets containing either 20 ppm PBB or no PBB, respectively (7). Ringer and Polin (8) showed that hatchability declined and chicks from contaminated eggs were less viable when hens were fed 125 ppm PBB in the diet, but not when fed 25 ppm (8) or 30 ppm (4). Quail hatched normally when PBB was fed at 25 ppm, but failed to hatch when 100 ppm (6).

The study reported herein will establish the relationship between PBB in eggs vs. hatchability by two approaches, which will be used to disagree on the extent of this relationship. The implications of this incompatibility may indicate that analysis for 6-BB might be a definitive approach to assess

PBB toxicity.

**Materials and methods.** Adult female White Leghorn chickens 10 months in production (about 60 weeks of age) were assigned at random into one of 7 treatments, or to a control group. Twenty-four hens were in each group. PBB, as Firemaster FF-1 was used in this experiment. This compound differs from that of Firemaster BP-6 used in other studies (2, 3, 6, 7) in that FF-1 has anti-caking substances added and had been milled to obtain a free-flowing compound. In other words, Firemaster BP-6 was an intermediate product. Firemaster FF-1 was the final product sold commercially, the one used in this study, and the chemical involved in the contamination of Michigan's livestock and poultry. It is reported (4) to contain 62.8% 6-BB and 13.8% 7-BB, as compared to 79.2% 6-BB and 14.3% 7-BB for the Firemaster BP-6 (2).

The details of the materials and methods used in the experiment, as well as the procedure for the analysis of PBB in eggs were reported (5). Briefly, the hens were fed FF-1 in the diet at 0.2, 1, 5, 25, 125, 625 or 3125 ppm for 5 weeks, then fed feed without FF-1 for 8 weeks to obtain data on withdrawal effects. Eggs require about 9-10 days to be completely formed, 8-9 of which are for yolk formation. Thus, sampling of eggs started on day 9 of the experiment, and was on every 7th day thereafter until the 37th day after withdrawal (Table I). The experiment started June 17, 1974. Starting on June 18th, eggs were saved. So that the time for egg sampling coincided with the middle of a 7-day collection period, the first setting of eggs in the incubator were those collected in the first 5 days on the experiment. All subsequent settings were from 7-day collections. The midpoint of the 1st collection period was day 9 on and represented equally days 6 through 8 for the accumulation phase and days 10 through 12 of the steady-state phase. Thus, the hatch value for a week's collection would represent the hatch value for the midpoint of

<sup>1</sup>Article No. 8433. Michigan Agricultural Experiment Station.

TABLE I.  
RELATIONSHIP BETWEEN HATCH AND HEXABROMOBIPHENYL (6-BB) IN EGGS

FIREMASTER FF-1 (FF-1) IN DIET - PPM															
		0.2		1.0		5.0		25.0		125.0		625.0		3125.0 <sup>A</sup>	
DATE	DAY FOR EGG SAMPLE	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH	6-BB IN EGG- PPM	% HATCH
6/27	DAY 9 ON	.14	(90) 92	1.8	(88) 93	7.7	(100) 93	23	(101) 91	232	(60) 28	-	(8) 0	-	-
7/4	16 ON	.26	(91) 95	1.4	(97) 92	11.0	(105) 97	85	(90) 87	304	(60) 5	-	-	-	-
7/11	23 ON	.34	(96) 88	1.3	(101) 84	3.4	(85) 89	46	(76) 96	178	(57) 2	-	-	-	-
7/18	30 ON	.43	(89) 96	1.5	(98) 89	5.6	(93) 95	33	(94) 85	145	(63) 0	-	-	-	-
7/25	2 OFF	.54	(62) 94	1.7	(93) 93	9.5	(86) 94	30	(82) 84	220	(50) 18	-	-	-	-
8/1	9 OFF	.58	(83) 92	0.8	(81) 91	1.9	(81) 94	11.3	(76) 92	58	(66) 74	-	-	-	-
8/8	16 OFF	.12	(74) 92	0.33	(75) 93	1.3	(61) 90	10.9	(61) 92	30	(58) 86	-	-	120	-
8/15	23 OFF	.10	(75) 93	0.25	(67) 84	0.83	(71) 94	6.8	(60) 87	54	(67) 85	78	-	65	(14) 43
8/22	30 OFF	.05	(64) 91	0.13	(60) 88	0.69	(52) 92	6.0	(57) 90	21	(55) 91	71	(18) 33	40	(17) 35
8/29	37 OFF	.07	(56) 95	0.13	(56) 96	0.84	(53) 91	5.3	(41) 83	19	(55) 78	73	(19) 21	48	(14) 21
9/5	44 OFF	-	(53) 93	-	(56) 100	-	(40) 98	-	(38) 92	-	(40) 93	-	(30) 37	-	(8) 63
9/12	51 OFF	-	(65) 94	-	(59) 95	-	(44) 91	-	(41) 83	-	(49) 88	-	(43) 19	-	(6) 67

( ) = NUMBER FERTILE; % HATCH = (NUMBER HATCH/NUMBER FERTILE) x 100

A = FF-1 WITHDRAWN 7 DAYS SOONER, THEREFORE ADD 7 DAYS TO "DAY FOR EGG SAMPLE"

Hatchability of eggs from White Leghorn chickens fed diets with polybrominated biphenyl. Firemaster FF-1, and the hexabromobiphenyl levels (6-BB) analyzed in eggs representative of each hatch.

that week; in this case, day 9 on. The eggs from the mid-point of the week were opened, pooled, and analysed for 6-BB by the Michigan Department of Agriculture, as previously described (5). Hens were artificially inseminated once a week with semen collected from males housed in a separate room and fed diet without FF-1.

6-BB was assayed by gas liquid chromatography using one or both procedures employing a <sup>3</sup>H-foil electronic detector at a temperature of 220° in the column and detector, and 250° in the injector, or a <sup>63</sup>Ni-detector at temperatures of 270° in the column, 310° in the detector and 300° in the injectorport. The important aspect for this experiment was that FF-1 was assessed from chromatograms by reading the peak height of the 6-BB peak using Firemaster BP-6 (Lot-#5143) as a standard. Subsequent comparison of this standard with those used by the Food and Drug Administration (FDA) showed comparable

patterns. The standard BP-6 was obtained from Michigan Chemical Company, the former manufacturer of FF-1. Linear and curvilinear regression and analysis of variance were applied to the data (9), after converting percentage values of hatch to arcsin  $\sqrt{\%}$  (9).

**Results and discussion.** Table I contains the weekly hatchability data (number hatch per number fertile) and the 6-BB levels of eggs representative of the day and week that the eggs were collected. Not included in Table I were the hatchability data for the first 5 days on the experiment for which no egg samples were obtained. These hatch values were 95.3, 80.0, 83.1, 88.1, 88.5, 90.0, 69.7 and 61.5% for the eggs from FF-1 levels of 0, 0.2, 1, 5, 25, 125, 625 and 3125 ppm in the diet, respectively. When these values were considered with those of Table I for the first 5 weeks that FF-1 was fed, hatchability of control eggs averaged 89.9 ( $\pm$  4.1)%, mean ( $\pm$  SD), and 91.2 ( $\pm$  3.1)% for the entire 13 weeks of the

experiment. Hatchability during 5 weeks of feeding FF-1 at 0.2, 1, 5 and 25 ppm were 90.6, 88.5, 92.8, and 89.9%, respectively; none of these values were significantly different,  $P \leq .05$ , from the control value. On the other hand, poor hatches were obtained when 125 ppm FF-1 was fed, but not until the hatch representing day 9 on (actually days 6-12) was obtained. Within the first 5 days of feeding FF-1 at 625 and 3125 ppm, hatchability was significantly ( $P \leq .05$ ) below normal. None of the eight fertile eggs hatched that were obtained from the hens fed 625 ppm PBB and representing day 9 on; and no eggs were laid by those hens fed 3125 ppm FF-1. A subsequent experiment revealed (4, 5) that during the steady-state phase of days 9 to 35 for feeding FF-1, the minimum effective level for FF-1 in the diet to produce a significant effect on hatchability was between 30 and 45 ppm.

6-BB was detected in whole egg samples, based on the dose-response curve for steady state values (5), at 0.3, 1.5, 7.4, 43.4, and 215 ppm for the treatment levels of 0.2, 1, 5, 25 and 125 ppm FF-1 in the diet, respectively. The latter level produced a high mortality in chick embryos (Table I) during the last few days of hatch. Edema of the abdominal and cervical regions was the prevalent pathological sign observed in embryos and newly hatched chicks from FF-1 treatment (Fig. 1a and 1b). The clinical signs resemble those of embryos from polychlorinated biphenyl treatment (7). The edema was the only side effect to be observed that was increased in incidence above abnormalities detected in control embryos.

Estimated  $t_{1/2}$  values were obtained from the 6-BB data in Table I and found to be 10 and 21 days for depletion time from prior treatment with FF-1 at 0.2-1.0 ppm, and



FIG. 1. Edematous condition of embryo (1b) and chick (1a) from feeding polybrominated biphenyl, Firemaster FF-1, to hens at dietary levels higher than 42 ppm. Note the accumulation of fluid typically seen in abdominal and head region of embryos, and cervical area of chicks.

TABLE II.<sup>a</sup>

Level of FF-1 withdrawn	Depletion curve	t <sub>1/2</sub>
0.2 ppm	$Y = -0.2024 - 0.0317X$	9.5
1.0 ppm	$Y = -0.0647 - 0.0286X$	10.5
5.0 ppm	$Y = 0.3287 - 0.0137X$	22.0
25.0 ppm	$Y = 1.1870 - 0.0131X$	23.0
125.0 ppm	$Y = 1.8818 - 0.0158X$	19.1

<sup>a</sup> Dose-response lines based on depletion curves for 6-BB from chicken eggs after removal of diets with FF-1. The relationship is  $Y = a + bx$ , where  $x$  = days of withdrawal starting at day 9 off, and  $Y$  = log ppm 6-BB in whole egg.

5–125 ppm, respectively (Table II). Thus, higher levels of FF-1 treatment required a longer time for depletion based on 37 days of measurements during withdrawal. Furthermore, factorial analysis of these depletion data revealed a significant linear and quartic, but not cubic, effect. This suggests that the slopes of the depletion curves are flattening to some extent and that with depletion beyond 37 days the  $t_{1/2}$  values will be greater.

As previously pointed out (5), the relationship between FF-1 in the diet and 6-BB levels in eggs during the steady-state phase, was expressed by the equation  $Y = 0.1763 - 1.012X$ , where  $X = \log$  ppm of FF-1 in the diet,  $Y = \log$  ppm 6-BB in whole egg. This is in good agreement with the data by other investigators (3, 6). The response of hatchability to FF-1 in the diet (3), during the steady-state phase, was estimated to be  $Y = 297.14 - 140.74X$ , where  $X = \log$  ppm FF-1, and  $Y = \arcsin \sqrt{\%}$  of hatchability.

The algebraic summation of these two regressions derived to relate log ppm 6-BB in egg, as  $X$ , to  $\arcsin \sqrt{\%}$  hatch, as  $Y$ , is given in Figure 2, line "a", along with a plot of the values from Table I for treatments with FF-1 at 25, 125 and 625 ppm. The regression lines under comparison are: (a) the line based on the derived steady-state values, (b) the line based on the depletion phase ("off" data), (c) the line based on the steady-state phase ("on" data), (d) the line representing both the steady-state and depletion phase of the data from Table I, and (e) a regression line based on egg residues between 30 and 85 ppm of 6-BB.

The lines for "b", "c", and "d" above were calculated on the basis of  $Y = a + bx + cx^2$ . From these regressions, a linear regression

can be calculated to represent the linear portion of these curvilinear lines by fitting the data into response lines for either "c", or "d" revealed slopes and intercepts very unlike the derived equation, "a" whose linear slopes were very similar (legend). The line calculated for "e" and a non-significant,  $P > 0.05$ , slope indicated by the lack of correlation ( $r$  = between hatchability and 6-BB levels = 0.85 ppm in eggs).

Considering all of these comparisons derived regression, the conclusion is reached that the derived equation relating hatchability to egg residues is not valid. Interestingly, the range of 6-BB levels from 30 to 85 ppm supposedly covered a range of hatchability from no effect down to 64%, based on the derived equation.

Fries *et al.* (10) reported that 7-BB depletes more rapidly in chicken eggs than 6-BB withdrawal of diets with BP-6. Also noted that the concentrations of these compounds in fat of hens being fed 20 ppm 4 times that of diet for 6-BB and 1.1 times that of diet for 7-BB. Thus, there is ev-

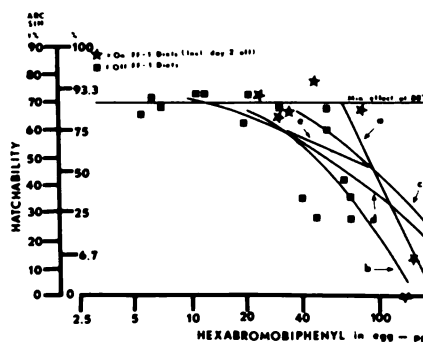


FIG. 2. Relationship between hexabromobiphenyl (6-BB) in whole egg and the hatchability of eggs during and after the feeding of diets with 25, 125 and 625 ppm Firemaster FF-1, a polybrominated biphenyl. Where  $X = \log$  ppm 6-BB and  $Y = \arcsin \sqrt{\%}$  hatchability, the response lines are: (a) A derived equation  $Y = 321.66 - 139.07X$ ; (b) a response line for data from the depletion phase where  $Y = 24.0 + 95.8X - 48.1X^2$ ; (c) a response line for data from the steady-state phase where  $Y = 96.8 + 10.7X - 18.8X^2$  whose linear portion is described by  $Y = 173.4 - 65.8X$ ; (d) summation of "b" and "c" where  $Y = 56.8 + 37.6X - 23.3X^2$  whose linear portion is  $Y = 167.2 - 67.3X$ ; and (e) a line for egg samples with 6-BB between 30 and 85 ppm where  $Y = 127.6 - 43.6X$ .

for differential metabolism of the isomers that comprise BP-6, and thus FF-1. The supposition to consider is that not all isomers of these PBBs gave equivalent toxicity and that this would account for our inability to find a close correlation between 6-BB and embryo toxicity over that wide range of 6-BB levels in eggs, and the incompatibility between derived and actual curves of 6-BB in eggs vs. hatchability. On this basis, these hatch and residue data indicated that analysis for FF-1 based on the analysis of only the 6-BB peak was not a definitive approach toward assessing toxicity of BP-6 or FF-1.

Other isomers, and their metabolites will have to be considered in the overall relationship of ingested PBBs to the residues in tissues and their toxicity of the compounds.

**Summary.** A relationship between polybrominated biphenyl, Firemaster FF-1 (FF-1), in the diet, and eggs, as monitored by hexabromobiphenyl (6-BB), and embryo toxicity, as measured by hatchability, were examined. The minimum dietary level of FF-1 for an effect on hatchability was estimated at 42 ppm, which produced an egg residue estimated at 65.9 ppm 6-BB. Generally, as FF-1 in the diet increased, egg residues increased and hatchability decreased. Regression equations were established for these relationships. After withdrawal of FF-1 in the diet, hatchability returned to normal when FF-1 treat-

ments had been <625 ppm. Over a range of 30–85 ppm 6-BB in eggs there was poor correlation to an effect on hatchability. These latter data were discussed in terms that the 6-BB peak may not be a definitive approach to assess FF-1 toxicity.

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Intraerythrocyte pH and Physiochemical Homogeneity<sup>1</sup> (40299)

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Intraerythrocytic pH is a major determinant of glycolytic metabolism, membrane function, and oxygen dissociation. We have investigated the possibility that in a physiochemical sense these functions are controlled by separate pH environments within the red cell.

Caldwell (1), Adler *et al.* (2), and Waddell and Bates (3), have demonstrated that the results of the determination of intracellular pH ( $pH_i$ ) by the distribution of a weak base differs from that determined by the distribution of a weak acid in a heterogeneous system. Using a weak acid, the  $pH_i$  closer to the highest pH of the various intracellular compartments is recorded, while a weak base reflects a value closer to the lowest pH of the compartments. If the  $pH_i$  as determined by a weak acid and a weak base are identical, then the cell interior is likely to be homogeneous (1). Using rat diaphragm muscle Adler (4) showed that the  $pH_i$  measured by the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO) was significantly higher than the  $pH_i$  measured by the weak base, nicotine. Physiochemical inhomogeneity, therefore, was demonstrated, as one might expect on morphological grounds. Using the same weak acid and weak base, we have investigated the  $pH_i$  of the human erythrocyte.

**Methods.** Venous blood was mixed with 14.3  $\mu$ g of heparin per ml, centrifuged, and the buffy coat removed. The erythrocytes were resuspended in their own plasma.

Radioactive DMO, 5,5-dimethyloxazolidine-2,4-dione-[2-<sup>14</sup>C] (New England Nuclear, Boston, MA), specific activity 11

mCi/mmole, was added to give a final concentration of 0.00066 mg/ml of blood. active nicotine, nicotine-methyl-[<sup>14</sup>C] linckrodt Corp., St. Louis, MO), specificity 2.41 mCi/mmole, was added to one aliquot of blood to give a final concentration of 0.011 mg/ml. Both tubes were incubated at 37° for 20 min, a time determined experimentally as adequate for both DMO and nicotine to reach equilibrium. The pH,  $pH_i$ , of each aliquot was measured to the nearest 0.01 unit using a Corning 165 pH blood gas analyzer (Corning Scientific Instruments, Medfield, MA). The aliquots were centrifuged, the plasma removed, and a microhematocrit corrected for the plasma [1.31% (5)] determined on the red blood cell packs. One ml of packed cells or one ml of plasma containing either nicotine-<sup>14</sup>C or DMO-<sup>14</sup>C was added to 5 ml of distilled water. Each sample was prepared in triplicate. One ml of each mixture was added into 2.0 ml of 10% trichloroacetic acid (TCA), centrifuged, and 1 ml of the supernatant counted in a Packard Tri-Carb Scintillation Counter Model 3320. Counts per minute were converted to disintegrations per minute by use of a quench curve.

**Binding studies.** To investigate the binding of DMO to human plasma, the method of Dryer (6) was utilized. One ml of normal heparinized (14.3  $\mu$ g/ml) plasma was incubated with 0.01 ml of DMO-[<sup>14</sup>C] at 37° for 30 min and an aliquot taken on to a G-25 Sephadex column with a void volume after balancing the column with DMO-[<sup>14</sup>C] in heparinized (15  $\mu$ g/ml) phosphate buffered saline. Aliquots of each fraction were counted and the optical density determined at 280 nm.

Binding studies of nicotine to human plasma and erythrocytes were also conducted. Ten ml of heparinized venous blood was incubated with 0.3 mg of nicotine-[<sup>14</sup>C] in 1 ml of 0.9% sodium chloride while another

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ml were allowed to incubate with the same amount of radioactive nicotine plus 0.85 mg of cold nicotine contained in 50  $\mu$ l of 0.9% sodium chloride. Three determinations were made from each tube of the ratio of intracellular to extracellular dpm per g of water.

**Calculations.** The external dpm for both DMO and nicotine determinations were converted to dpm/g of plasma water using 0.94 as the fraction of solvent water in plasma (7).

The internal dpm for both DMO and nicotine determinations were converted to dpm/g of erythrocyte water after correction for the trapped plasma in the erythrocyte pack, utilizing 0.59 as the fraction of solvent water in the erythrocyte (8-11).

The dpm/g of plasma water and cell water are then entered into the appropriate formula (12).

$$\text{pH}_i = \text{pK}'_a + \log \left[ \frac{(\text{DMO})_i (10^{\text{pH}_e - \text{pK}'_a} + 1) - (\text{DMO})_e}{(\text{DMO})_e} \right]$$

The  $\text{pK}'_a$  of DMO is 6.13 (12).

$$\text{pH}_i = \text{pK}'_a - \log \left[ \frac{(\text{nicotine})_i (10^{\text{pK}'_a - \text{pH}_e} + 1) - (\text{nicotine})_e}{(\text{nicotine})_e} \right]$$

The  $\text{pK}'_a$  of nicotine was chosen as 7.85, the value used by Effros and Chinard (13).

The  $\text{pH}_e$  used was the average of the two measured values, which never differed by more than 0.01 of a pH unit. There was no significant difference between the  $\text{pH}_e$  values measured in the tube containing DMO versus the tube containing nicotine.

**Results.** The DMO and plasma binding study showed no rise or subsequent fall in dpm occurring in association with the collection of the plasma protein peak. Thus, there was no evidence of binding of DMO to human plasma.

The studies done to evaluate the possibility of nicotine binding to human plasma or erythrocytes showed that the ratio of internal to external dpm per g of water was identical at the two widely different concentrations of nicotine. This is strong evidence against binding or active transport of nicotine as well as evidence against the permeability of the erythrocyte membrane to the nicotine ion (14).

Table I shows the results of the  $\text{pH}_i$  deter-

minations using both DMO and nicotine. Four determinations using each indicator were carried out for each normal sample. The range of the four values falls within 0.16 of a pH unit. There is no significant difference by analysis of the 5 pairs of DMO and nicotine results.

**Discussion.** Caldwell (1) stated that if the values for  $\text{pH}_i$  obtained from multiple different indicator methods are in agreement it is probable that the cell interior is "reasonably uniform". Waddell and Bates (3), using a current operational definition of  $\text{pH}_i$ , stated that in an inhomogenous system,  $\text{pH}_i$  calculated from the distribution of a weak acid yields a pH value closer to the higher value, and that  $\text{pH}_i$  calculated from the distribution of a weak base yields a result closer to the lower pH value in that inhomogenous system.

Accurate determinations of  $\text{pH}_i$  using such indicators depend upon the absence of binding, the absence of active transport, and the impermeability of the cell membrane to the ionic species of the weak acid or weak base used. Waddell and Butler (15) demonstrated that DMO is not significantly bound to bovine serum albumin. Calvey (16) showed that DMO is not bound to, or actively transported by, rabbit erythrocytes. Bromberg *et al.* (17) showed that human erythrocytes do not bind DMO. We have demonstrated that DMO is not bound to human plasma and that nicotine meets the three criteria for accurate indicator compounds set forth above.

We have chosen 0.59 gm  $\text{H}_2\text{O}/100$  ml of

TABLE I.  $\text{pH}_i$  (AVERAGE APPEARS ABOVE THE RANGE) DETERMINED BY DMO AND NICOTINE IN HUMAN ERYTHROCYTES.

Sample	$\text{pH}_e$	DMO	Nicotine
A	7.325	7.06 6.98-7.14	6.99 6.98-7.03
B	7.360	7.12 7.07-7.17	7.04 7.01-7.08
C	7.335	7.15 7.08-7.20	7.09 7.07-7.12
D	7.355	7.09 7.06-7.12	7.11 7.09-7.14
E	7.325	7.09 7.07-7.12	7.05 6.99-7.14
No significant difference <sup>a</sup>			

<sup>a</sup> Calculations based on paired data analysis on five pairs.

cells as the fraction of solvent water in the erythrocyte. This value is amply supported (10-13). However, values up to 0.72 gm H<sub>2</sub>O/100 ml of cells (9) can be defended. Use of the latter value would assume no bound water and would produce a DMO value 0.1 of a pH unit lower and a nicotine value 0.1 of a pH unit higher than the average values we calculated. Such a result would be at variance with the theoretical considerations presented by Waddell and Bates (3).

In performing the above experiments we used erythrocytes of various ages. Assuming that each cell is homogeneous but that they vary somewhat in intracellular pH as a function of age, the pH<sub>i</sub> determined by DMO should be equal to the pH<sub>i</sub> measured by nicotine.

There are only two results possible if a weak acid and a weak base are used to determine the pH<sub>i</sub>. Either the weak acid gives a higher pH<sub>i</sub> reading than the weak base, in which case the system is heterogeneous, or the readings are equal, in which case the system is homogeneous. Our experiments utilized erythrocytes from four normal donors and the same indicator compounds, DMO and nicotine, that were used to demonstrate physiochemical heterogeneity of skeletal muscle cells (4). Our results show no significant difference between the pH<sub>i</sub> value obtained by DMO and that obtained by nicotine. It is possible that in a heterogeneous system pH<sub>i</sub> values determined by these compounds could, fortuitously, turn out identical, as has been suggested by Carter (18) in his report of equal pH<sub>i</sub> values determined by DMO and nicotine in barnacle muscle, an apparently heterogeneous system. We have no proof that this has not occurred here but it would seem unlikely given the difference between the erythrocyte and barnacle muscle fiber microscopically. Further, Bone *et al.* (19) using a single donor showed no significant difference between the hydrogen-ion concentration in erythrocytes as determined by the weak acid DMO and the weak base ammonia.

We conclude that the internal pH of the normal human erythrocyte is uniform throughout the cell and that this cell is physiochemically homogeneous. This information supports the concept that in the human erythrocyte hydrogen-ion dependent processes

such as glycolytic metabolism, mercuric function and oxygen dissociation are related by a single value for each cell.

**Summary.** In order to determine the physiochemical homogeneity of the human cell, intraerythrocyte pH was simultaneously measured using the weak acid 5,5-dimethyl-oxazolidine-2,4-dione (DMO) and the weak base nicotine. If a cell is homogeneous, measurements will yield the same result. If a cell is heterogeneous, the DMO reading will be closer to the highest pH in the cell while the nicotine will read closer to the lowest pH. The results show no significant difference between the intracellular pH obtained by either of these methods (average DMO = 7.10, by nicotine = 7.06 at a constant external pH of 7.33). We conclude that the human erythrocyte is physiochemically homogeneous.

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## Stimulation of Erythropoietin Secretion by Single Amino Acids (40300)

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in deprivation in rats results in a decrease of the amount of erythropoietin (Ep) and in response to hypoxic stimulation this effect is rapidly reversed if the protein-deprived animals are fed a single protein consisting of albumin or hemoglobin prior to or shortly after the onset of the stimulus (2). The study detailed here provides evidence that in protein-deprived rats erythropoietin secretion can be stimulated by individual amino acids.

**Materials and Methods.** Female Sprague-Dawley rats weighing 100–200 g were used. The control diet in pellet form containing less than 5% protein ("protein-free"), but otherwise nutritionally complete, was purchased from Purina-Ralston Co., Missouri. Solutions of amino acids in 3 cc of distilled water were injected into the rat stomach. To determine the effect of amino acids, the pH of the solution was changed at times from moderately acidic (pH 3.0) to strongly alkaline (pH 9.0). However, in each individual experiment, the pH of the water fed to control rats was adjusted to that of the test amino acid solutions. No difference in the pH of the various solutions fed to the rats was not found to affect plasma Ep levels.

Erythropoietin production was stimulated in the animals in a hypobaric chamber by exposing them to 0.5 atmosphere for 24 hr. Immediately afterwards, rats were sacrificed by cardiocentesis and the plasma obtained from each experimental group (4–5 rats) was pooled and assayed for erythropoietin levels in posthypoxic mice by the method of Gordon and Straub (3) (6–8 assay mice each receiving 0.5 ml of pooled plasma). It should be noted that minor variations in the spring mechanism of the regulatory valve of our hypobaric chamber result in some inconsistencies in the chamber pressure from experi-

ment to experiment. Therefore, a control group of rats fed water adjusted to the pH of the amino acid preparations were always included in each experimental trial. The statistical significance of the differences was determined by the Student's *t* test.

**Results. Effect of single L-amino acids (Table I).** Rats fed a protein-free diet for 6 days were fed 100 mg of an L-amino acid and were immediately afterwards exposed to hypoxia. Table I shows the mean plasma Ep levels of rats fed various amino acid solutions. The data indicate that L-methionine, L-cystine and L-leucine produced the most intense and consistent stimulation of Ep production ( $P < 0.001$ ). L-Tyrosine and L-asparagine produced a small but still significant ( $P < 0.05$ ) increase in plasma Ep levels whereas the rest of the amino acids had no significant effect ( $P > 0.05$ ).

**Effect of various doses of L-amino acids (Tables II and III).** Protein-deprived rats fed from 25 to 100 mg L-methionine or L-cystine prior to hypoxia had significantly higher plasma Ep levels compared to the control group. Increasing the amount fed to 800 mg did not produce a further increase in Ep production and may have been inhibitory. When histidine or glycine was fed in doses of 10 to 400 mg per rat, no increase in the posthypoxic plasma Ep levels was detected.

**Discussion.** Decreased Ep production occurs in the presence of protein deficiency (1). This decrease has been related by some to the depression of basal metabolism associated with starvation (4). We have recently demonstrated that a single feeding of protein (hemoglobin or albumin) to protein-deprived rats produces an immediate enhancement of Ep production which is dose related and which does not correlate with changes in the oxygen consumption of the animals (2). We concluded that the production of erythropoietin depends not only on oxygen supply vs

TABLE I. EFFECT OF FEEDING A SINGLE L-AMINO ACID ON POSTHYPOXIC PLASMA Ep LEVELS OF PROTEIN DEPRIVED RATS.

Amino acid fed (100 mg)	% <sup>59</sup> Fe uptake into RBC's of assay mice (mean $\pm$ 1 SEM)	
	Control (H <sub>2</sub> O) group	AA group
Alanine (3)	2.83 $\pm$ 0.65	3.94 $\pm$ 1.00
Arginine (3)	2.83 $\pm$ 0.66	4.04 $\pm$ 1.27
Valine (3)	4.75 $\pm$ 1.10	5.97 $\pm$ 1.70
Serine (4)	4.32 $\pm$ 0.79	3.00 $\pm$ 0.98
Methionine (7)	3.08 $\pm$ 0.33	11.00 $\pm$ 1.19 <sup>a</sup>
Cystine (4)	2.79 $\pm$ 0.73	10.10 $\pm$ 0.60 <sup>a</sup>
Tyrosine (3)	1.96 $\pm$ 0.16	4.74 $\pm$ 0.98 <sup>b</sup>
Tryptophane (2)	2.55 $\pm$ 0.87	1.61 $\pm$ 0.18
Phenylalanine (2)	2.86 $\pm$ 0.56	2.41 $\pm$ 0.28
Leucine (5)	2.06 $\pm$ 0.12	4.52 $\pm$ 0.44 <sup>a</sup>
Isoleucine (2)	2.27 $\pm$ 0.03	4.29 $\pm$ 1.11
Histidine (4)	3.16 $\pm$ 0.55	3.34 $\pm$ 0.32
Asparagine (4)	2.18 $\pm$ 0.07	4.84 $\pm$ 0.51 <sup>b</sup>
Glycine (6)	3.98 $\pm$ 1.01	5.95 $\pm$ 1.58
Lysine (4)	3.76 $\pm$ 1.12	4.29 $\pm$ 1.38
Glutamic acid (4)	3.56 $\pm$ 1.25	6.99 $\pm$ 2.10
Aspartic acid (4)	3.56 $\pm$ 1.25	5.62 $\pm$ 1.53
Threonine (4)	4.12 $\pm$ 1.00	6.90 $\pm$ 1.99
Proline (5)	4.93 $\pm$ 1.17	8.77 $\pm$ 2.92
Cysteine (6)	3.50 $\pm$ 0.69	5.34 $\pm$ 0.99

Numbers in parentheses signify the number of experimental trials conducted.

<sup>a</sup>  $P < 0.001$ .

<sup>b</sup>  $P < 0.05$ .

TABLE II. EFFECT OF FEEDING VARIOUS DOSES OF L-METHIONINE AND L-CYSTINE ON POSTHYPOXIC PLASMA Ep LEVELS OF PROTEIN DEPRIVED RATS.

Amount fed	% <sup>59</sup> Fe Incorporation into RBC's of assay mice (mean $\pm$ 1 SEM)	
	L-Methionine	L-Cystine
H <sub>2</sub> O	2.09 $\pm$ 0.25	2.74 $\pm$ 0.23
25 mg	8.55 $\pm$ 4.61	4.20 $\pm$ 1.41
50 mg	8.41 $\pm$ 3.39	7.00 $\pm$ 2.05
75 mg	10.02 $\pm$ 3.90	4.91 $\pm$ 1.07
100 mg	10.43 $\pm$ 1.96	5.79 $\pm$ 0.23
H <sub>2</sub> O	3.68 $\pm$ 0.74	1.50 $\pm$ 0.21
100 mg	12.67 $\pm$ 1.05	9.19 $\pm$ 0.94
400 mg	12.15 $\pm$ 1.09	6.56 $\pm$ 1.41
800 mg	5.70 $\pm$ 0.27	5.41 $\pm$ 0.32

demand of the Ep producing sites, but also on the continuous supply of amino acids (2). The present experiments were done to determine whether individual amino acids were important for the biosynthesis of erythropoietin as occurs with other polypeptide hormones (insulin, growth hormone) (5). Only three amino acids, (methionine, leucine and cystine) had a significant effect in raising the

TABLE III. EFFECT OF FEEDING VARIOUS DOSES OF L-HISTIDINE OR L-GLYCINE ON POSTHYPOXIC PLASMA Ep LEVELS OF PROTEIN DEPRIVED RATS.

Amount fed	% <sup>59</sup> Fe Incorporation into RBC's of assay mice (mean $\pm$ 1 SEM)	
	L-Histidine	L-Glycine
H <sub>2</sub> O	4.11 $\pm$ 0.47	3.18 $\pm$ 0.27
10 mg	4.00 $\pm$ 0.60	3.66 $\pm$ 0.23
50 mg	2.51 $\pm$ 0.58	4.63 $\pm$ 0.56
100 mg	4.24 $\pm$ 0.50	4.51 $\pm$ 0.75
400 mg	3.32 $\pm$ 0.40	2.18 $\pm$ 0.21

plasma Ep levels of the protein deprived rats. Other amino acids had minimal or no effect. There is a parallel to this finding in the studies which show a great variation in the ability of single amino acids to stimulate secretion of insulin or growth hormone (6, 7).

Some amino acids which had no effect at the 100 mg dose level were tested at smaller doses to determine whether their dose response curves were maximal at the lower levels. The results were negative. The data also suggest that higher doses of cystine and methionine may be inhibitory. We have no explanation for this possibility, although large doses of amino acids may suppress the transport of other amino acids across cell boundaries (8).

**Summary.** Protein deficiency in rats results in decreased ability to produce erythropoietin after hypoxic stimulation. This defect can be reversed by a single protein feeding at the time of exposure to hypoxia. The present experiments show that feeding of methionine, leucine or cystine also corrected the defect in erythropoietin production. These amino acids may serve to signal the adequacy of protein reserves and permit the synthesis of erythropoietin. Other single amino acids had minimal or no effect.

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## Ornithine Decarboxylase Activity in Cells Acutely and Chronically Transformed by Murine Sarcoma Virus (40301)

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Polyamine biosynthesis is one of the earliest events occurring during cellular proliferation (1). Ornithine decarboxylase (ODC), which catalyzes the formation of putrescine from ornithine, is the rate limiting enzyme in polyamine biosynthesis (2). Resting cells have low, stable ODC levels which increase rapidly upon the onset of growth (3, 4). We have demonstrated that increased ODC activity follows infection of cultured mouse Balb/3T3 (B/3T3) cells with murine sarcoma virus (MSV) (5). The increase in ODC activity is independent of the population doubling time and commences immediately prior to morphological transformation. Elevation of ODC levels also precedes morphological transformation by Rous sarcoma virus (6).

Transforming stocks of MSV consist of mixtures of defective transforming virus and non-transforming murine leukemia virus (MuLV) (7). The MuLV is usually present in great excess, and dual infection of mouse cells with both viruses is required for MSV replication. Cells infected with the transforming virus alone undergo transformation, and retain the sarcoma genome, but do not release infectious virus. Two such classes of transformed cells have been described: (a) Non-producer (np) which do not release virus particles (8), and (b) sarcoma virus positive, leukemia virus negative (S+L-) cells which release noninfectious virus particles and have MuLV gs antigen (8). Superinfection of both of these transformed cell classes with MuLV results in release of infectious transforming and nontransforming viruses. However, superinfected S+L- cells undergo further morphological alteration (thus providing a focus assay for MuLV). Superinfection of np cells does not result in morphological alteration.

In this communication we describe experiments studying the relationship between elevated ODC activity, virus induced morphological transformation, virus production, and rates of cellular division. We compare producer, np and S+L- derivatives of a single murine cell clone.

**Materials and methods.** Cell lines. B/3T3, clone A31, is a contact inhibited, 'flat' non-virus releasing cell (9). It becomes transformed after MSV infection, but productive infection with MuLV does not induce morphological change. D245E6 is a S+L- B/3T3 clone selected for its relative 'flatness'. After MuLV superinfection, its morphology becomes more transformed (10). KA31 is a Kirsten MSV transformed np clone of B/3T3 (11). MuLV superinfection of KA31 results in release of transforming and nontransforming viruses without morphological change. B/3T3 and KA31 cells were obtained from Dr. Stuart Aaronson and D245E6 cells from Dr. Robert Bassin. Cells were maintained in 75 mm flasks in 5% CO<sub>2</sub> atmosphere at 37°. Fluids were changed at 24 or 48 hr intervals. Cells were grown in Eagle's essential medium (D245E6) or Dulbecco's modification of it (B/3T3 and KA31). Medium was supplemented with 10% heat inactivated (56°, 30 min) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml).

**Viruses and virus assays.** Gz-MSV, a mixture of transforming and nontransforming viruses, was recovered from the supernatant fluids of acutely infected B/3T3 cells, and had a titer of  $2 \times 10^6$  focus forming units/ml (12). Rauscher leukemia virus, a strain of MuLV, was obtained by concentrating the supernatant fluids of chronically infected BALB/c JLSV-9 cells, and had a titer of  $1 \times 10^7$  plaque forming units/ml. Infectious center assays were modifications of the commonly used methods for assays of MSV and MuLV (8, 13). Fifty or 100 mitomycin C

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d (25  $\mu$ g, 1 hr) test cells were added onto already seeded indicator cells. For MSV assays the indicator cells were B/3T3, and foci refractile MSV transformed cells were enumerated 5 days later. For MuLV assays, A1-2 cells (14) were used as indicator and plaques (consisting of supertransformed cells that had lysed or floated away) enumerated 5 days later. Colony forming efficiency (CFE) in semi-solid medium was determined by suspending  $1 \times 10^5$  viable cells in 1% agarose over a 0.9% agarose base.

1, 8 and 15 days, another layer of tissue was placed over the cell containing colonies. Colonies were counted 18 days after infection.

**Methods.** ODC activity was assayed by measuring enzyme released  $\text{CO}_2$  as described previously (5). Replicate plates were incubated twice with saline and frozen ( $-20^\circ$ ) and assayed. Cells were gently scraped into 1 ml, freeze-thawed three times, and centrifuged (4500g for 10 min). Supernatant fluids (0.5 ml) were incubated with 50  $\mu$ l [ $^{14}\text{C}$ ]line in plastic tubes equipped with a Teflon stopper supporting a polyethylene bead. After incubation ( $37^\circ$ , 45 min), 0.1 ml hydroxide of hyamine was added to each well. After a further incubation of 15 min, 0.2 ml of perchloric acid was added to

each well. Tubes were agitated for 15 min to release bound  $\text{CO}_2$ , the center wells were removed, and their radioactivities determined. Protein was determined by the Lowry method (12). Cells were counted with a hemocytometer, and viability determined by trypan blue exclusion.

**Results.** Properties of the cell lines used are presented in Table I. Uninfected B/3T3 cells were epithelioid and contact inhibited, did not release virus, and failed to grow in soft agarose. Productive infection with MuLV did not alter its morphology. Within 48 hr of MSV infection, B/3T3 cells became round or spindle shaped, were highly refractile and adhered poorly to the substrate. Morphological transformation was accompanied by release of transforming and nontransforming viruses, and the ability to grow in soft agarose at low efficiency. Uninfected D245E6 cells were large polygonal cells with slight overlapping of their edges, which grew in soft agarose but did not release infectious virus. On superinfection with MuLV, D245E6 cells underwent further morphological transformation, and closely resembled MSV infected B/3T3 cells. The superinfected cells released both MSV and MuLV, but their ability to grow in soft agarose decreased. Uninfected KA31 cells were small and highly refractile,

TABLE I. CHARACTERISTICS OF CONTROL AND VIRUS-INFECTED CELLS.

Cell line	Transformed morphology <sup>a</sup>	% Infectious Centers <sup>b</sup>		% CFE in soft agarose	Maximum ODC activity (pmoles/ $10^6$ cells) <sup>c</sup>
		MuLV	MSV		
F3	0	<0.1	<0.1	<0.1	49
F3 + MuLV	0	78	<0.1	<0.1	63
F3 + MSV	+++	100	54	0.8	995
5 + MuLV	+	<0.1	<0.1	16	77
5 + MuLV (transfer 0)	+++	6	9	0.3	509
5 + MuLV (transfer 4)	+++	7	11	5	145
5 + MuLV (transfer 0)	+++	<0.1	<0.1	15	179
5 + MuLV (transfer 4)	+++	94	83	7	356
5 + MuLV (transfer 0)	+++	45	38	3	165

<sup>a</sup> Untransformed morphology arbitrarily graded as follows: 0 contact inhibited, nonrefractile cells similar to parent; + nonrefractile cells with some cellular overlapping; ++ refractile cells with formation of dense cellular foci; +++ highly refractile cells with scant cytoplasm and poor anchorage dependency, the cells tended to float to the supernatant fluid prior to reaching confluency.

<sup>b</sup> Percent foci 5 days after plating control or infected cells on A1-2 (MuLV assays) or B/3T3 monolayers (MSV assays).

<sup>c</sup> Maximum ODC activity is the highest measured level of enzyme activity, usually occurring four days after infection.



with short spindly processes. They grew in soft agarose before and after superinfection with MuLV, but released MuLV and MSV only after superinfection.

Growth characteristics of the cells employed are shown in Fig. 1. The growth rates of uninfected B/3T3 and D245E6, and MuLV infected B/3T3 were similar, while uninfected and infected KA31 cells grew more rapidly and reached a higher cell density at day 7. As noted previously (5) MSV transformed B/3T3 cells grew slower than uninfected cells, although the differences were not marked in the present experiment, when tissue culture grown virus stocks were substituted for animal tumor harvests. MuLV superinfection of D245E6 cells resulted in a considerable increase in the population doubling time, but had no effect on the growth of KA31 cells. Trypan blue exclusion studies revealed less than 2% nonviable cells in control and virus infected cell lines at all observation points.

ODC levels of cell lines were measured 1, 3, 4, 5 and 7 days after seeding. The highest levels measured (usually occurring 4 days after seeding) are shown in Table I, and the entire curves are presented in Fig. 2. Relatively low ODC levels occurred after seeding and at confluence. Comparable data were obtained when ODC activity was expressed as a function of cell number or of cellular protein. Control B/3T3 and D245E6, and MuLV infected B/3T3 had relatively low 'maximum' levels (ie. the highest levels measured). MSV infection of B/3T3 and MuLV infection of D245E6 cells resulted in 20- and sevenfold increases respectively in maximum ODC activities. Uninfected KA31 cells had

a higher baseline ODC activity than the other cell lines, but superinfection resulted in a twofold increase only.

The temporal relationships between elevation of ODC activity, morphological transformation and virus production were also studied. After four passages MSV transformed B/3T3 and MuLV superinfected D245E6 and KA31 cells had not further altered morphologically, or in their ability to release transforming and non transforming viruses, or grow in soft agarose (Table I). However, ODC levels of MSV infected B/3T3 cells fell rapidly after transfer (Fig. 2), while the drop in ODC levels of superinfected D245E6 cells was smaller and took longer. ODC levels of superinfected KA31 cells fell only slightly, but the baseline levels were high and the initial rise on superinfection was modest.

Individual clones of B/3T3 and MSV transformed B/3T3 cells were selected after isolation in liquid or semi-solid media. Characterization of the 13 clones selected and their ODC data are presented in Table II and Fig. 3. Uninfected B/3T3 clones (numbers 1-5) had a flat morphology, did not release viruses, failed to grow in soft agarose, and had low maximum ODC levels. MSV transformed clones 6-12 had higher ODC levels, which appeared related to the degree of morphological transformation. Although isolated from MSV infected cells, clone 13 released only MuLV, had a flat morphology, did not grow in soft agarose, and had low ODC activity. Presumably this clone arose from a cell infected with the nontransforming component of MSV only. With one exception, all clones had maximum ODC activities 4 days after

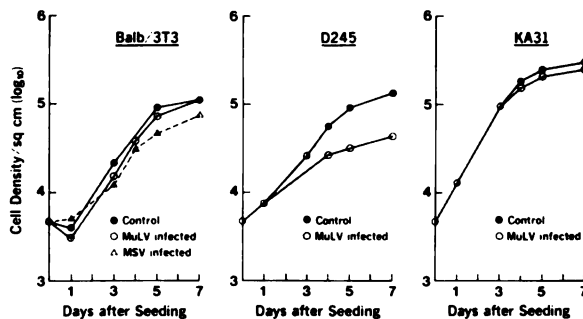


FIG. 1. Growth curves of uninfected and virus-infected cell lines. Cells were infected in suspension with MuLV at a multiplicity (MOI) of 3:1 or MSV (MOI 10:1) at 37° for 1 hr prior to seeding.

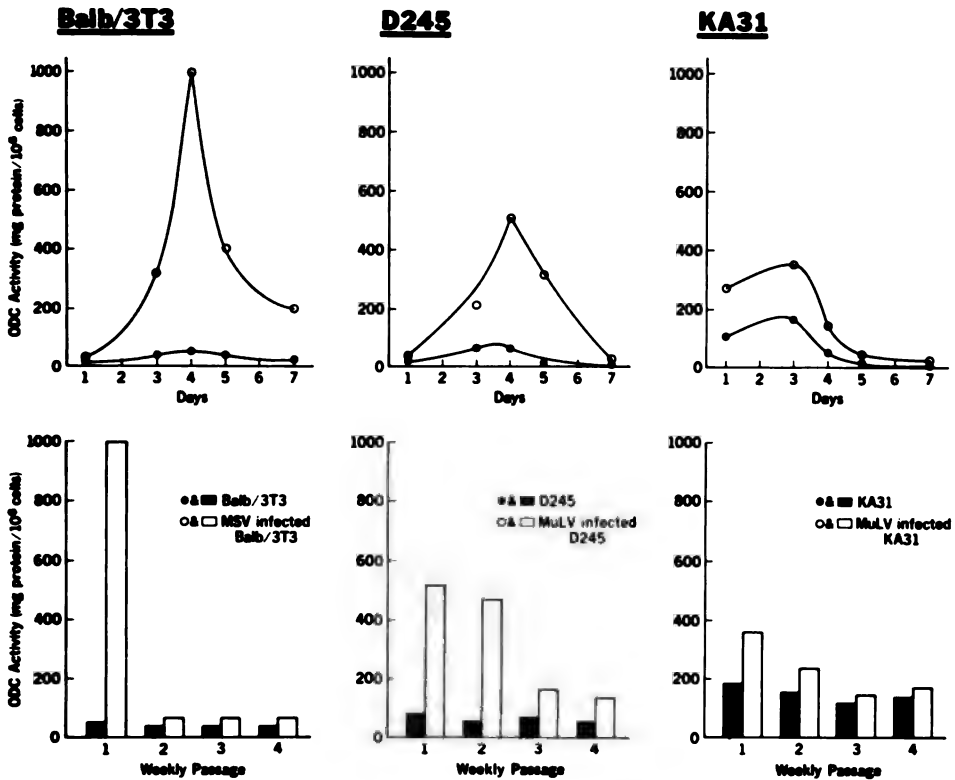


FIG. 2. ODC activity of control and transformed Balb/3T3 cells. In the upper panel, cells were infected immediately prior to seeding and harvested on days 1, 3, 4, 5, and 7. ODC activities in bar graphs (lower panel) represent the maximum levels measured during weekly cell passages.

TABLE II. CHARACTERISTICS OF CONTROL, TRANSFORMED AND VIRUS-INFECTED BALB/3T3 CLONES.<sup>a</sup>

Clone #	Transformed morphology	% Infectious centers		% CFE in Soft agarose	Maximum ODC activity (pmol/10 <sup>6</sup> cells)
		MuLV	MSV		
1	0	<0.1	<0.1	<0.1	66
2	0	<0.1	<0.1	<0.1	79
3	0	<0.1	<0.1	<0.1	74
4	0	<0.1	<0.1	<0.1	27
5	0	<0.1	<0.1	<0.1	31
6	+	90	100	0.1	36
7	+	100	100	31	104
8	+++	100	100	0.5	570
9	+++	100	100	0.1	901
10	+++	100	24	69	582
11	+++	100	100	1	522
12	+++	100	76	31	493
13	0	100	<0.1	<0.1	45

<sup>a</sup> Uninfected B/3T3 clones (#1-5) were isolated from liquid medium. MSV transformed clones were obtained on liquid (#7-12) or semi-solid media (#6 and 13). Clones were transferred 18 days after seeding, and analyzed 30 days later. See also legend of Table I.

eding. The exceptional clone divided slower than the others and was still in exponential growth phase at day 7.

**Discussion.** While previous studies have in-

dicated that virus induced transformation results in increased intracellular ODC levels, the relationship is complex. Our present experiments further define the association by

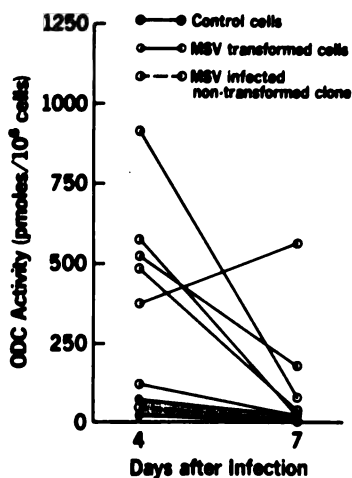


FIG. 3. ODC Activity of control and MSV infected Balb/3T3 Clones. The data of the control clones with the highest and lowest ODC values are displayed. The remaining three control clones had intermediate values lying within the shaded areas. One clone isolated from the MSV infected culture (○—○) was nontransformed and only released MuLV. The ODC values of one MSV transformed clone fell within the shaded area and is not represented.

studying several parameters, including morphology, growth rate, virus production and time. ODC levels of transformed and non transformed cells alter with cell growth, the highest levels occurring during logarithmic growth. Elevation of ODC levels (during cell growth) accompanies cellular morphological change to a transformed or more transformed phenotype. Following infection, B/3T3 manifests considerable changes in both morphology and ODC activity, D245E6 more modest alterations, and KA31 essentially none. The elevated ODC levels accompanying transformation cannot be explained by increases in cell growth rates; doubling times (B/3T3 and D245E6) are lengthened or unaltered (KA31) after virus infection. ODC elevation is also not related to release of transforming or non transforming viruses. The cloning experiments indicate that the relatively few transformed clones so obtained have higher ODC levels (during cell growth) than non transformed clones. While transformed clones have a wide range of ODC activities, acute virus transformation is consistently accompanied by a very high elevation. With cell passage, ODC levels of acutely transformed

cells return towards baseline levels, perhaps because most acutely transformed cells fail to divide.

Our findings that elevated ODC levels accompany acute virus induced morphological transformation may be explained by the recent report of Isom (16). She found that infection of fibroblasts by potentially oncogenic human cytomegalovirus (CMV) rapidly induced a multiplicity dependent increase in ODC activity. Isom's experiments indicate that CMV infection overrides end product repression of ODC by putrescine. Thus the oncogenic potential of a virus may be related to its ability to interfere with normal regulatory functions of key cellular metabolic enzymes.

**Summary.** Ornithine decarboxylase (ODC) activity increases when cells are acutely transformed with murine sarcoma virus (MSV). Three contact inhibited or MSV transformed clones of Balb/3T3 were transformed or supertransformed by MSV or its accompanying non-transforming 'helper' virus (MuLV), and the relationships between ODC activity, morphology, virus production and growth rates were examined. Clones isolated from these lines were also studied. All of the virus infected lines released both MSV and MuLV. ODC activities could not be correlated with differences in growth rates. The only consistent relationship was between elevated ODC activity and acute morphological transformation, suggesting that polyamine metabolism plays a crucial role in the transformation process. With time, the elevated ODC activities returned towards baseline levels. Thus ODC activity does not appear to be a useful marker for chronic infection or transformation by type C viruses.

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# Suppressed Dietary Inducibility of Glucose 6-Phosphate Dehydrogenase and E Cyclic AMP in Acute Hepatic Injury<sup>1</sup> (40302)

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Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) is a key enzyme of the pentose phosphate pathway and induced by dietary glucose and amino acids, but not by either alone (1-3). Thus, the dehydrogenase level in rat liver is under a dual dietary control, *i.e.* transcriptional and posttranscriptional regulations; a glucose-dependent step of the induction being sensitive to actinomycin D (3) and blocked by increasing cyclic 3',5'-adenosine monophosphate (cyclic AMP) level (4). An entirely different type of G6PD induction could be brought about by intoxication of rat with carbon tetrachloride and other hepatotoxins (5-7). Although the synthesis *de novo* of the enzyme protein is involved in the hepatotoxin-induced increase in G6PD activity, it does not require newly synthesized RNA (5) and is insensitive to manipulations to raise hepatic cyclic AMP level (8). We found in our preliminary experiments with acute thioacetamide intoxication of rat that the dietary induction of G6PD was markedly depressed in the injured liver (9).

A further study of this observation, reported in the present communication, revealed that the reduced dietary inducibility of G6PD in the acute hepatic injury could be explained at least by a dietary unresponsive increase in cyclic AMP level in the injured liver. Thioacetamide was chosen in this study to produce an acute liver damage with elevated G6PD activity, because the intoxication with thioacetamide, unlike carbon tetrachloride, caused no reduction in dietary intake.

**Materials and methods.** Male Sprague-Dawley rats, weighing 130-150 g, were deprived of food for 24 hr before intraperitoneal injection of 20 mg thioacetamide (Merck Co., Darmstadt, Germany; dissolved in saline) per

100 g body weight. The animals were fasted for 24 hr and divided into the following three groups: GC, refed on a glucose (7:3 in weight) mixture; G, placed on a glucose diet; and S, starved for additional 24 hr. Control rats received equivalent amount of saline in place of the thioacetamide and treated identically with respect to the dietary change.

The animals, each group consisting of 10 rats, were killed at indicated time intervals after ether overdose (10), which gives a high level of cyclic AMP and cyclic 3',5'-guanosine monophosphate (cyclic GMP) values both to those obtained by a freezing method (11). A small portion of the liver was removed, weighed (10) and extracted by fixing with trichloroacetic acid (12). After removal of TCA with ether, the aqueous extract was evaporated under nitrogen and reconstituted in water to give an original volume. The cyclic nucleotide concentrations were determined, after appropriate dilution and succinylation, by radioimmunoassay method using cyclic AMP and cyclic GMP kits (Shoyu Co., Chiba, Japan) (13).

The activities of G6PD, low-K<sub>m</sub> glucose 6-phosphate dehydrogenase (EC 2.7.1.1) and glucokinase (EC 2.7.1.2) in liver supernatants and of aminotransferase (GPT, EC 2.6.1.2) and the contents of glycogen in liver were determined as described previously (7, 14). The enzyme activities and cyclic nucleotide concentrations in liver were expressed on the basis of unit supernatant protein, because the liver weight increased markedly in refed groups of rat due to an excessive glycogen deposition (Table I). All the data are given as means and standard error of means for each group. Histological examinations were made on liver specimens by a hematoxylin-eosin staining.

**Results.** Alterations of G6PD activity

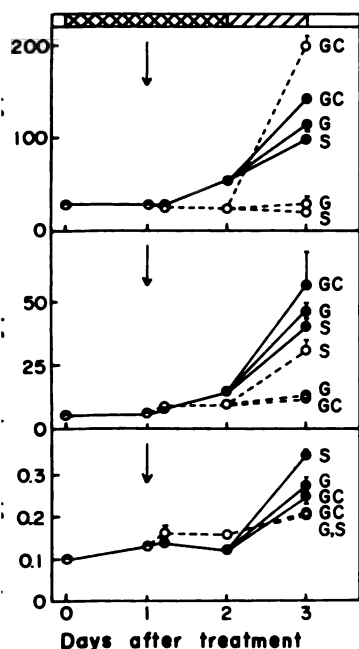
<sup>1</sup> This work was supported by a Grant-In-Aid for Scientific Research (B) from the Ministry of Education, Science and Culture.

cleotide contents in liver following and thioacetamide treatments are illustrated in Fig. 1 and those of other parameters of hepatic injury and dietary effect are listed in Table I. The specific activity of cyclic AMP in control animals increased markedly on refeeding with glucose and casein, with glucose alone. In thioacetamide-treated rats, G6PD activity increased significantly at 24 and 48 hr of the hepatotoxin treatment even if the animals were starved. Necrosis and regeneration of hepatocytes were evident histologically. These data are in agreement with our previous findings (1-3, 5). The starved and thioacetamide-treated rats placed on a glucose-casein diet, only showed a further increase in G6PD activity induced by the refeeding (GC vs. G or S) in contrast with the steep rise found in the control rats. There was no difference in the amount of diet consumed between the injured and control groups. The results apparently

indicated that the dietary induction of G6PD was impaired in the injured liver despite the fact that the enzyme activity was increased by hepatic injury itself. That the extent of hepatic injury *per se* was not affected by the different dietary treatments was evident from the similar increases in low-Km hexokinase activity in the three different dietary groups of injured rats (Table I). The hepatic level of this enzyme increases by liver injury (6, 9, 14) and is unresponsive to dietary change as the data for control groups given in the same table reveal. There were no significant differences either in serum GPT activity, a sensitive marker of liver injury, among the three injured groups (Table I). The activity of glucokinase, another dietary inducible enzyme (1, 15), was reduced by thioacetamide intoxication and the induction of this enzyme by glucose or glucose-casein refeeding was also diminished in the injured livers as may be seen in the table.

The values of cyclic AMP obtained with livers of well-fed rats (0-day value in Fig. 1) fell in the range of reported values (10-12, 16). The hepatic cyclic AMP level increased significantly in 3 days of starvation in both thioacetamide-injured and control groups, although the extent of the increase was slightly larger in the injured group than in the control. An important result of this experiment is that the rise in hepatic cyclic AMP content on prolonged fasting of injured rats could not be suppressed by refeeding glucose-containing diets in contrast with the rise in the control animals. The hepatic levels of cyclic GMP in control groups agreed well with the reported values (11, 12) and changed little by dietary alteration. In thioacetamide-treated rats, however, the cyclic GMP content increased significantly upon prolonged starvation. The increase was much less, although above the control levels, in the refed groups of intoxicated rats. A possibility of overestimating cyclic GMP level in the presence of high concentrations of cyclic AMP was neglected by obtaining constant values with different dilutions of liver extract in radioimmunoassay.

In thioacetamide-treated rats, the amount of glycogen deposited in the liver after refeeding was significantly less than in untreated rats, even though dietary intakes were



Activities of G6PD and levels of cyclic AMP and cyclic GMP in livers of control and thioacetamide-treated rats placed on different dietary regimens. The arrow indicates the time of administration of thioacetamide (●) or saline (○---○). XXXXXX, period of fasting for all the groups; and XXXXXX, period of continued fasting for Groups G and GC.

TABLE 1. ACTIVITIES OF GPT IN SERA AND OF GLUCOKINASE AND LOW-K<sub>m</sub> HEXOKINASE IN LIVERS AND CONTENTS OF GLYCOGEN AND PROTEIN IN LIVERS OF CONTROL AND THIOACETAMIDE-TREATED RATS PLACED ON DIFFERENT DIETS.

Experimental conditions		Enzyme activities			Tissue constituents	
Dietary manipulation	Thioacetamide treatment	GPT	Glucokinase	Hexokinase	Glycogen	Supernatant protein
		(Karmen u./ml)	(nmoles/min/mg protein)		(mg/g liver)	
Well-fed	—	32 ± 2	20.3 ± 4.2	3.2 ± 1.3	42.0 ± 7.7	96 ± 2
1-day starved	—	24 ± 1	8.9 ± 0.4	4.4 ± 0.3	3.6 ± 3.6	113 ± 2
1-day and 5 hr starved	—	27 ± 2	10.7 ± 1.8	3.4 ± 0.4	1.9 ± 0.8	115 ± 4
	+ (5 hr)	24 ± 4	9.1 ± 0.6	5.0 ± 0.5	1.1 ± 0.5	108 ± 2
2-day starved	—	21 ± 3	10.6 ± 1.8	3.2 ± 0.2	0.2 ± 0.1	110 ± 4
	+ (1 day)	224 ± 53	6.2 ± 1.7	10.2 ± 0.4	0.2 ± 0.1	103 ± 3
3-day starved	—	32 ± 6	2.5 ± 0.6	3.0 ± 0.2	0.2 ± 0.1	123 ± 3
	+ (2 days)	261 ± 66	0.4 ± 0.3	20.1 ± 1.4	0.2 ± 0.1	102 ± 2
2-day starved and 1-day refed on G	—	21 ± 3	27.5 ± 2.2	3.1 ± 0.2	69.4 ± 7.1	80 ± 4
	+ (2 days)	323 ± 100	9.0 ± 2.2	21.8 ± 1.3	35.8 ± 8.4	88 ± 2
2-day starved and 1-day refed on GC	—	29 ± 4	41.9 ± 2.7	3.9 ± 0.2	80.0 ± 9.8	84 ± 4
	+ (2 days)	229 ± 50	7.6 ± 2.7	20.9 ± 0.6	31.5 ± 16.1	86 ± 1

similar in both groups of rat and almost no ingested dietary mass remained in the gastrointestinal tracts at the time of sacrifice.

**Discussion.** G6PD is a unique enzyme in a sense that a single molecular species is involved in a wide variety of inductive responses; such as those to dietary, hepatotoxic and neoplastic changes (17). Thus, the induction mechanism of this enzyme appears to be different depending on the type of inductive stimuli. The dietary induction of G6PD requires *de novo* RNA synthesis at a low cyclic AMP level (3, 5), whereas carbon tetrachloride-induced increase of G6PD synthesis obligates neither of them (7). The latter mechanism would also apply to the increased hepatic G6PD level in thioacetamide-injured rat (17). Accordingly, the impairment of dietary induction of G6PD in injured liver is possibly at the level of transcription. The block at this step could be accounted for at least by the high hepatic level of cyclic AMP observed in the thioacetamide-injured rats refed on glucose and casein. The increased level of cyclic AMP appears to be also responsible for the reduced accumulation of hepatic glycogen in the injured refed rats. Incidentally, the low hepatic cyclic AMP level alone is not sufficient to induce this enzyme, since in control rats a sole glucose diet lowered the cyclic AMP level without inductive effect.

Whether the dietary unresponsive increase in cyclic AMP level by thioacetamide treatment is due to a sustained hyperglucagonemia or an altered adenylate cyclase-phosphodiesterase system is to be solved in future studies. Although an increased portal level of glucagon is reported in acute ethionine intoxication of rat, glucose infusion has been shown to decrease the hepatic cyclic AMP content (16). Prostaglandin may well be an attractive candidate for such a stimulant as to the dietary insensitive elevation of cyclic AMP in injured liver.

A reduced dietary response of G6PD in regenerating liver following partial hepatectomy (18) could be similarly explained by elevated cyclic AMP levels in the remnant liver (10). Since, however, the thioacetamide-induced hepatic degeneration and necrosis is also followed by a rise in DNA synthesis (7), some conditions associated with cell division may serve as another common underlying mechanism for the suppression of dietary induction of G6PD. The small increases in hepatic cyclic GMP content found in the late stage of thioacetamide injury might be more or less related to the regenerative process of the injured liver (7, 19), although a direct effect of the carcinogen can not be excluded (20).

It is of some interest to note that another dietary inducible and cyclic AMP-sensitive

ne, glucokinase (1, 15), was also shown to be less responsive to glucose-containing diets in the injured liver. Since G6PD and  $\alpha$ -hexokinase could be induced by hepatic injury itself (5, 6, 9, 14), the decrease in kinase activity may also represent a specific metabolic response of hepatocyte to the injury rather than a mere destructive process of general protein synthesis. Thus, in hepatic injury, the induction of more differentiated enzymes is suppressed and that of primitive or fundamental enzymes is enhanced, resulting in an undifferentiated enzyme pattern (6, 14). A similar loss of dietary response to G6PD and other carbohydrate-metabolizing enzymes in preneoplastic livers has been demonstrated by Poirier and others (21). An increased inducibility of some enzymes of glucose acid metabolism in chronic administration of carbon tetrachloride and a noncarcinogenic azo dye is also reported from their study (22). Although thioacetamide is a hepatocarcinogen, its acute intoxication, as employed in the present experiment, could be interpreted better as a hepatic injury, which has little significance as precancerous lesion. Elucidation of the mechanisms of altered enzyme induction in acute hepatic injury would provide a clue for the understanding of undifferentiated gene expression in neoplastic livers and in turn hepatomas.

**Summary.** The dietary induction of liver G6PD was found to be markedly impaired in acute hepatic injury of rat caused by thioacetamide intoxication. The level of cyclic AMP in the injured liver was increased and could not be reduced by glucose-containing diets. The results indicated that the suppressed dietary inducibility of G6PD in hepatic injury is accounted for at least by the dietary unresponsive increase in cyclic AMP in the injured liver.

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## Blood Volume Changes during the First Week after Birth in the Beagle and Pig (40303)

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Birth marks the end of the parasitic and aquatic life of the fetus and the beginning of numerous physiological adjustments which adapt the newborn to a new and different environment. Among the adjustments which occur in different species at birth are those involving the circulatory system, and within this system are the changes in red cell and plasma volumes and venous hematocrit. However, data available for the newborn human over the first few days following birth present no clear pattern of change in the plasma volume, red cell volume, blood volume, or hematocrit. A portion of the variability in these data may be the result of early or late clamping of the umbilical cord (1, 2). But even if analysis of the data is restricted to those investigations where the cord is clamped early, the results are contradictory. Plasma volume, for example, is reported to remain constant over the first 24 hr following birth (3), to increase significantly within 3-5 hr (4, 5), to decrease in the first 2½ hr (6), or to increase over the period of 4-24 hr after birth (1). Changes reported for red cell volume, blood volume, and hematocrit are equally varied, although in most of these investigations blood volume and red cell volume are calculated from the measured plasma volume and hematocrit.

There are relatively few studies examining the changes in blood volume immediately after birth for species other than the human. In the pig, McCance and Widdowson (7) report a 30% increase in plasma volume 24 hr following birth, while Ramirez *et al.* (8) report a small but significant increase in blood volume during the first 12-hr period following birth. In the rat there is a small reduction in plasma volume between days 4 and 14 (9), but, contrary to the results of these authors, in the same species Garcia (10) reports a rise in plasma volume from birth to 15 days of age, and Constable, no significant change (11).

In the present article, data are presented for both the pig and the beagle for the period between birth and day 7 following birth, and the changes in plasma, red cell, and blood volumes and hematocrits are examined.

*Materials and methods.* The beagles used in this investigation were from the colony maintained at the Wynne Unit of The Texas Department of Correction in Huntsville. A description of the physical facility and the routine procedures used for breeding, immunization, and diet was published previously (12). A pig colony for research purposes was established while one of us (R.H.) was serving as acting chairman of the Department of Physiology at Mahidol University in Bangkok, Thailand, and was maintained at Kasetsart University by the courtesy of university officials and The Rockefeller Foundation. Details of the management of this colony also have been published (13). Standard procedures, modified for small animals, were used to measure red cell volume with <sup>51</sup>Cr (14) and plasma volume either with <sup>131</sup>I-albumin or the dye T-1824. There was no statistically significant difference between the plasma volumes measured with <sup>131</sup>I-albumin or T-1824 (12, 15). Hematocrits were measured by the micro method; no correction was made for trapped plasma. Beagle pups up to 3 hr after birth were not sedated, while those older than 4 hr were given 0.5-1 mg of morphine sulfate, injected subcutaneously. The pigs were anesthetized with 5-10 mg/kg of pentobarbital sodium, administered intravenously. Different animals, and usually from the same litter, were used for the collection of data for each of the time periods after birth.

*Results.* The data for the beagles are presented in Table I. For day 0 (day of birth) data were available from 10 min to 18 hr following birth, and because there was evidence of significant changes within this time, the data were divided into three 6-hr periods. The average age of the pups was 2.5 hr for

TABLE 1. RED CELL AND PLASMA VOLUME CHANGES IN NEWBORN BEAGLES.

After birth	Body weight kg	Red cell volume ml/kg	Plasma volume ml/kg	Blood volume ml/kg	Venous hematocrit %	Circulatory hematocrit <sup>a</sup> %	BVR cells <sup>b</sup>
0 hr	0.190 ± 0.02 <sup>d</sup> (12) <sup>c</sup>	49.2 ± 2.6 (12)	46.4 ± 1.5 (4)	95.6 ± 4.9 (5)	56.0 ± 1.9 (11)	51.0 ± 1.9 (5)	0.906 ± 0.02 (5)
2.5 hr	0.244 ± 0.005 (4)	38.7 ± 2.3 (4)	45.3 ± 0.3 (4)	84.0 ± 2.6 (4)	52.3 ± 2.6 (4)	45.9 ± 1.3 (4)	0.881 ± 0.02 (4)
		<i>P</i> < 0.05 <sup>f</sup>					
8.5 hr	0.266 ± 0.01 (4)	40.5 ± 1.0 (4)	53.3 ± 1.3 (4)	93.6 ± 2.0 (4)	47.5 ± 1.5 (4)	43.2 ± 0.4 (4)	0.912 ± 0.03 (4)
			<i>P</i> < 0.001	<i>P</i> < 0.05			
16.5 hr	0.275 ± 0.01 (20)	45.4 ± 1.9 (20)	48.2 ± 1.1 (13)	91.3 ± 2.5 (13)	53.5 ± 1.4 (20)	47.1 ± 1.2 (13)	0.899 ± 0.01 (13)
Day 1	0.251 ± 0.01 (10)	56.7 ± 4.5 (10)	62.2 ± 3.1 (10)	118.9 ± 2.5 (10)	52.1 ± 2.7 (10)	47.3 ± 2.9 (10)	0.892 ± 0.01 (10)
		<i>P</i> < 0.02	<i>P</i> < 0.001	<i>P</i> < 0.001			
Day 2	0.273 ± 0.006 (10)	46.2 ± 4.0 (10)	59.1 ± 2.2 (10)	105.3 ± 4.6 (10)	46.4 ± 2.6 (10)	43.3 ± 2.3 (10)	0.933 ± 0.02 (10)
		<i>P</i> < 0.05		<i>P</i> < 0.05			
Day 7	0.436 ± 0.21 (10)	35.6 ± 2.2 (10)	62.2 ± 2.6 (8)	97.8 ± 3.1 (10)	38.8 ± 1.5 (10)	36.4 ± 2.2 (10)	0.930 ± 0.01 (10)
		<i>P</i> < 0.001			<i>P</i> < 0.001		

<sup>a</sup> cell volume/(red cell volume + plasma volume).

<sup>b</sup> ulatory hematocrit/venous hematocrit.

<sup>c</sup> age time.

<sup>d</sup> n ± SE.

<sup>e</sup> umber of animals.

<sup>f</sup> ue for difference from previous value.

at 6-hr period, 8.5 hr for the second and 16.5 hr for the last 6-hr period. mean red cell volume for beagles 2.5 was 49.2 ± 2.6 ml/kg. In pups 8.5 hr red cell volume was significantly less (.05), and did not change again during at 6-hr period. The plasma volume of .5 hr old was 46.4 ± 1.5 ml/kg, with nge during the next 6 hr; however, it nificantly higher (*P* < 0.001) in pups r old. Blood volume was 95.6 ± 4.9 in pups 2.5 hr after birth and decreased s 8.5 hr old due to the decrease in red lume. The blood volume, as the result nificant increase in plasma volume n 8.5 and 16.5 hr, was only slightly 16.5 hr than at birth. The venous xcrit decreased during the successive 6- ods, resulting in a significantly (*P* < ower hematocrit in pups 16.5 hr old r those at 2.5 hr. The trend for the tory hematocrit was the same as that venous hematocrit; consequently, the f circulatory to venous hematocrit was ally unaltered.

cell and plasma volumes were signifi- higher for the day-1 than for the day-

0 pups, using the pooled data for the 20 beagles on day 0. This increase in plasma and red cell volumes resulted in a significant increase in blood volume (*P* < 0.001) for the day-1 pups. On day 2 there was a significant decrease in red cell volume and blood volume (*P* < 0.05), but only a slight reduction in plasma volume. On day 7 there was a further significant decrease (*P* < 0.001) in red cell volume, while the decrease in blood volume was not significant due to an increase, although not significant, in plasma volume.

Changes in venous and circulatory hematocrits reflected those of cell and plasma volumes throughout the period of study. Since the increases in red cell and plasma volumes between day 0 and day 1 were of the same magnitude (20–23%), neither circulatory nor venous hematocrit changed significantly, and the ratios of the two hematocrits (BVR<sub>cells</sub>) remained the same. From day 1 to day 2 both venous and circulatory hematocrits decreased, but not significantly; however, between days 2 and 7 there was a further significant decrease in venous hematocrit accompanied by a similar change in circulatory hematocrit. The BVR<sub>cells</sub> remained relatively

constant over the first 7 days after birth ( $0.899 \pm 0.01$  on day 0 and  $0.93 \pm 0.01$  on day 7), indicating that there was no shift in the distribution of red cells and plasma in the circulation during the 7-day period.

For the pig (Table II), the exact times at which red cell and plasma volumes were measured on day 0 were not known; therefore, only the mean value was calculated. The principal changes observed in the pig were an increase in plasma volume ( $P < 0.05$ ) between days 1 and 2 and a decrease in red cell volume between days 0 and 2 and days 2 and 7, with the decrease on day 7 significant when compared with day 0 ( $P < 0.05$ ). Blood volume decreased progressively, and on day 7 it was significantly less than that measured on day 0 ( $P < 0.05$ ). Venous hematocrit decreased between days 0 and 1 ( $P < 0.05$ ), with no further significant change on day 2 or day 7. The values for  $BVR_{\text{cells}}$  were 0.85 on day 0 and 0.79 on day 7.

**Discussion.** During the first few days after birth there are changes in both the red cell and plasma volumes in the beagle and the pig, but the pattern of the changes is different for the two species. What the red cell and plasma volume changes are in the human over the first few days after birth is uncertain at present because of the diversity of the data. However, data by Usher *et al.* (1) suggest that in the human neonate, as in the pig and beagle, there is over the first few days following birth an increase in plasma volume and a decrease in red cell volume and venous

hematocrit, although there are differences in the time relationship at which the changes occur.

In the beagle the increase in plasma volume, which may occur as early as 12 hr following birth, is accompanied by an increase in plasma protein concentration. The increase in total protein concentration is due to an increase in the globulin fractions, while the albumin concentration remains stable, so that the albumin-globulin ratio decreased significantly (15). Thus the expansion of plasma volume can be explained by a shift of fluid into the circulation due to an increase in plasma protein. This shift of fluid among body compartments is substantiated further by the finding in the beagle that between days 0 and 1 there is a significant increase in the volume of extracellular fluid, at the expense of intracellular fluid, while total body water remains constant (16).

An increase in plasma volume similar to that in the beagle occurs in the pig, although the increase is between days 1 and 2. According to McCance and Widdowson (7), who first observed an increase in plasma volume within hours following birth of the pig the increase is the result of absorption of colostrum through the gut with a marked increase in the globulin portion of total plasma protein concentration. This mechanism is suggested also as an explanation of the plasma volume expansion in the beagle.

In the immediate neonatal period, red cell volume in the beagle, but not in the pig, is

TABLE II. RED CELL AND PLASMA VOLUME CHANGES IN NEWBORN PIGS.

Time after birth	Body weight kg	Red cell volume ml/kg	Plasma volume ml/kg	Blood volume ml/kg	Venous hematocrit %	Circulatory hematocrit <sup>a</sup> %	BVR cells <sup>b</sup>
Day 0	$1.7 \pm 0.05^c$ (16) <sup>d</sup>	$24.8 \pm 2.1$ (12)	$63.2 \pm 2.1$ (11)	$88.0 \pm 3.2$ (9)	$33.1 \pm 1.3$ (16)	$28.1 \pm 1.4$ (11)	$0.85 \pm 0.04$ (11)
Day 1	$1.6 \pm 0.05$ (14)	—	$58.0 \pm 2.5$ (12)	—	$27.1 \pm 2.7$ (14)	—	—
Day 2	$1.5 \pm 0.14$ (8)	$20.2 \pm 1.4$ (7)	$66.7 \pm 3.3$ (6) $P < 0.05$	$86.9 \pm 3.4$ (5)	$26.6 \pm 1.3$ (8)	$23.0 \pm 1.2$ (6)	$0.86 \pm 0.03$ (6)
Day 7	$2.4 \pm 0.11$ (23)	$19.7 \pm 1.0$ (15)	$64.4 \pm 1.5$ (20)	$82.7 \pm 1.6$ (12)	$29.9 \pm 0.8$ (23)	$23.8 \pm 1.9$ (15)	$0.79 \pm 0.04$ (15)

<sup>a</sup> Red cell volume/(red cell volume + plasma volume).

<sup>b</sup> Circulatory hematocrit/venous hematocrit.

<sup>c</sup> Mean  $\pm$  SE.

<sup>d</sup> Number of animals.

<sup>e</sup> P value for difference from previous value.

le than the plasma volume, and, plasma volume, the changes are difficult. At 2.5 hr after birth the red cell volume is  $49.2 \pm 2.6$  ml/kg in the beagle, significantly over the next 6–18 hr, during the next 24 hr, then decreasing between 48 and 72 hr. The venous hematocrit reflects the changes in red cell and plasma volumes fairly consistently. For example,

on day 0 to day 1 there is a significant increase in both red cell and plasma volume as the percentage increase for the same, there is no change in the venous hematocrit. On day 2 there is a significant (19%) increase in red cell volume and a significant increase in plasma volume, and these changes are accompanied by an 11% fall in venous hematocrit. Inasmuch as the venous hematocrit is affected by shifts in both red cell and plasma volumes, it cannot be used to estimate changes in either red cell or plasma volume. Shifts in the circulatory hematocrit (whole body hematocrit) follow shifts in venous hematocrit, so that the difference between the two hematocrits remains approximately the same. Therefore, for the beagle during the first period after birth, it is possible to estimate either red cell or plasma volume by use of this ratio ( $BVR_{\text{cells}}$ ) and venous hematocrit, estimate the other volume with reasonable accuracy.

The red cell volume for newborn mongrel dogs is 135 ml/kg (17), than the 95.6 ml/kg measured for the beagle. This difference in data for the newborn dog results from two factors. The first factor is that (17) measured plasma volume and red cell volume from the venous hematocrit. The latter represents the venous hematocrit and therefore overestimates red cell and blood volumes. The second factor may be even more important in the estimation of blood volume for other species including the dog, the use of both  $^{131}\text{I}$ -tagged albumin and dye from the circulation is more reliable in the newborn than in the adult (15). Usually, with either of these tags, when red cell volume is calculated from a single sample, as was done in the newborn monitor, the time of sampling after the tag on day 0, the greater will be the error in the plasma volume measure-

ment. For instance, in pups on day 0 a sample taken 15 min after injection of the tag may overestimate plasma volume by 15%, resulting also in an overestimation of red cell and blood volumes.

The fluctuations in red cell volume in the beagle during the first week after birth pose several questions; one of them is the possible sites from which the red cells can be sequestered or released. The volume of red cells shifting into and out of the circulation is relatively large: between 2.5 and 8.5 hr after birth the circulating red cell volume decreases by 22%, while between 18 and 48 hr after birth red cell volume increases by 28%. Changes in red cell volume of a similar magnitude for the human over the first 5-hr period after birth are reported by Sisson and Whalen (18). The difference between their results (17) and those reported for other newborn humans (6) may be explainable on the basis of the time of cord clamping during birth. This explanation, however, does not appear to be applicable to the changes seen in the beagle. Sisson and Whalen (18) also postulated, as an explanation for the changes in red cell volume in the newborn human, "an initial temporary sequestration of blood in the viscera and caudal end of the body," and the blood was later "introduced into the general circulation as vascular and pulmonary patterns were stabilized."

The spleen and bone marrow are suggested also as blood reservoirs capable of significantly increasing blood volume in the human during the first 24 hr after birth (19). In the adult dog both the spleen and the liver are known to be active red cell reservoirs (20), but whether this is true also in the newborn pup can be inferred only from indirect data. The unit red cell volume (ml/100 g) of all organs in the beagle decreases between days 0 and 1 (21, 22). The combined red cell volume of the heart, lungs, kidneys, spleen, stomach, skeletal muscle, intestines, and skin is 28% of the total red cell volume on day 0, but decreases to 16% on day 1. The decrease in the volume of red cells in these tissues coincides with an increase in the circulating red cell volume on day 1. These data, while providing no information on the mechanisms concerned in the relatively rapid fluctuations in red cell volume of the newborn beagle, do

provide tentative support to the idea that there may be reservoirs of red cells in the circulation of the newborn and that red cells move in and out of these reservoirs under the control of unknown stimuli.

**Summary.** During the first week of post-natal life, there were significant changes in red cell volume, plasma volume, and venous and circulatory hematocrits in both the beagle and the pig. In beagle pups the mean red cell volume decreased between 2.5 and 8.5 hr after birth, then increased at 16.5 hr, with a further increase on day 1. Between days 2 and 7, red cell volume decreased. There was evidence of a release of red cells into the circulation from red cell reservoirs. In the newborn pig, red cell volume decreased between days 0 and 2, but was not significantly different on day 7 from day 2. In the beagle the mean plasma volume did not change during the first 12 hr following birth; it increased between 12 and 24 hr after birth and remained unchanged through day 7. In the pig, plasma volume decreased between day 0 and day 1, increased on day 2, and was not significantly different on day 7 from day 2. The increase in plasma volume was the result of an increase in plasma protein, which caused a redistribution of fluid among the various fluid compartments. In the beagle, blood volume decreased between 2.5 hr and 8.5 hr, increased at 16.5 hr with a further increase on day 1, then decreased on day 2, with no further change on day 7. The blood volume in the pig decreased progressively between day 0 and day 7. The changes in venous and circulatory hematocrits for both the beagle and pig reflected those of red cell and plasma volumes throughout the first week of life. The  $BVR_{\text{cells}}$  did not change significantly, indicating that there was no shift in the distribution of red cells and

plasma in the circulation over this 7-day period.

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# Time of Exposure to Estradiol and LHRH Effect LH Release From Bovine Pituitary Cells?<sup>1</sup> (40304)

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nizing hormone releasing hormone) induced increase in serum LH is coincident with periods of increased LH secretion in cows (1), ewes (2), (3, 4) and female rats (5, 6). Exogenous also increase magnitude of LH by LHRH in cows (7), ewes (8), (9) and female rats (10). However, studies to demonstrate direct effects of estradiol on LH secretion *in vitro* have yielded conflicting results, i.e. estradiol increased (11), decreased (13-15) or did not change (16), magnitude of LHRH induced LH release in

experiments reported, we investigated effects of estradiol and LHRH on LH secretion from bovine pituitary cells in primary culture. Variables were dose and time of exposure of cells to E<sub>2</sub> and LHRH alone or in combination.

**Materials.** Medium for culture was Dulbecco's minimal essential medium<sup>3</sup> supplemented with essential and non-essential amino acids and buffered as in reference 12. Solutions of synthetic LHRH<sup>3</sup>, prepared in 0.1% Knox gelatin:0.05 M phosphate buffered saline, were added to cultures in 10% Estradiol-17 $\beta$  (E<sub>2</sub>)<sup>3</sup> in 10% ethanol, added in volumes such that final concentration of ethanol in medium was 0.1%.

**Cultures.** Bovine pituitary cell cultures were prepared (12). Briefly, bovine anterior pituitaries were sliced ( $\approx$ 1 mm), diced ( $\approx$ 1 mm) and resulting pieces washed thrice with

medium. Pituitary cells were dispersed from these pieces by stirring in 0.3% collagenase<sup>3</sup> for 45 min then 0.25% Viokase<sup>3</sup> for 15 min. Washed cells were suspended ( $\approx$ 5  $\times$  10<sup>5</sup> cells/ml) in medium containing 10% bovine serum<sup>3</sup> and 1 ml of suspension transferred to each well of multiwell culture plates. Pituitary cells were in culture for 5 days with medium changed at 24-hr intervals beginning at 48 hr. On day 5 cells were washed 4 times with serum free medium and treatments begun. Medium did not contain serum during treatment.

**Experimental design. Experiment 1.** The objective was to determine effects of varying time of exposure and concentration of LHRH on quantity of LH released. Treatments were arranged as a five  $\times$  six factorial experiment with concentration of LHRH (0, 0.1, 1.0, 10 and 100 ng/ml) and time (.75, 1.5, 3, 6, 12 and 24 hr) as main effects. There were six replicates per treatment combination ( $n$  = 180).

**Experiment 2.** The objective was to determine effects of varying time of exposure and concentration of estradiol on quantity of LH released. Treatments were arranged as a three  $\times$  four factorial experiment with concentration of estradiol (0, 5 and 50 ng/ml) and time (3, 6, 12 and 24 hr) as main effects. There were 12 replicates per treatment combination ( $n$  = 144).

**Experiment 3.** The objective was to examine the interaction of estradiol and LHRH on LH release over time. Treatments were arranged as a four  $\times$  two  $\times$  five factorial experiment with concentrations of estradiol (0, 0.5, 5.0, and 50 ng/ml) and LHRH (0 and 100 ng/ml) and time of exposure to estradiol and LHRH (1.5, 3, 6, 12, and 24 hr) as main effects. There were four replicates per treatment combination ( $n$  = 160).

Within each experiment, treatments were begun concurrently and medium collected

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From all correspondence should be sent. Dulbecco's medium from Difco Labs, Detroit, MI; courtesy of Dr. R. Rippel, Abbott, N. Chicago, IL; estradiol 17 $\beta$  and collagenase (type I-150  $\mu$ /mg) from Sigma, Chicago, Ill.; Viokase from GIBCO, Grand Island, New York.

and frozen after the prescribed interval of treatment. Medium was assayed for LH by methods described in 18.

**Statistical analysis of data.** In instances where data, hormone concentrations or time were not normally distributed, statistical analysis were performed after logarithmic transformation of values. Data from each experiment were analyzed by analysis of variance appropriate to factorial experiments (19). Significant differences due to main effects were determined by Dunnett's t-test (20). Additionally, data were subjected to polynomial regression analysis (19) to evaluate change in LH release over time or concentration of hormones tested.

**Results. Experiment 1.** Effects of varying time of exposure and concentration of LHRH on LH concentration in medium are in Fig. 1. In the absence of LHRH, LH accumulated in medium during 24 hr and this increase was curvilinear ( $P < 0.001$ ) i.e. rate of accumulation increased with time. Within time periods, increase in LH release by LHRH over the range 0.1 to 100 ng/ml, was linear ( $P < 0.001$ ) when exposure was for .75, 1.5, 3, 6, or 24 hr but curvilinear ( $P < 0.001$ ) when for 12 hr. Dose-response slopes generated from data normalized by logarithmic transformation were not different among times i.e. with the exception of 12 hr, LH release induced by 100 ng LHRH/ml was twice that of comparable control values. However, the actual increase in amount of LH release (ng/ml) over controls, induced by each concentration of LHRH, increased with increasing time of

exposure.

**Experiment 2.** Effects of varying time of exposure and concentration of estradiol on quantity of LH in medium are in Table 1. Estradiol did not affect concentration of LH in medium when present for 3 hr but increased ( $P < 0.001$ ) LH relative to controls when present for 6, 12 or 24 hr. Both concentrations of estradiol tested increased LH accumulation in medium and magnitude of increase was dependent on the dose of  $E_2$  i.e. 50 ng  $E_2$  released more LH than 5 ng ( $P < .01$ ).

**Experiment 3.** Effects of varying time of exposure and concentration of estradiol on LHRH induced LH release are in Fig. 2. Within each combination of LHRH and estradiol, accumulation of LH in medium was curvilinear ( $P < 0.001$ ) and greater ( $P < 0.001$ ) for cultures incubated with LHRH than for comparable controls. Estradiol, present for 1.5 or 3 hr, did not affect LH concentration in medium of cultures incubated with or without LHRH. However, when estradiol was present 6, 12 or 24 hr LH accumulation in medium was increased ( $P < 0.001$ ) relative to controls. This was true for cultures incubated with or without LHRH. In addition, magnitude of LH release, within these time periods, was linearly ( $P < 0.001$ ) related to concentration of estradiol used. A comparison of cultures incubated with and without LHRH, within time, revealed that slopes of estradiol dose-response were not different ( $P > 0.10$ ).

**Discussion.** Results confirm our previous

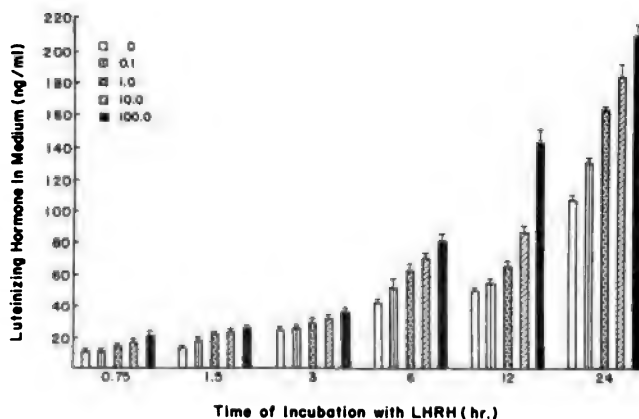


FIG. 1. LH concentration in medium following incubation of bovine pituitary cells with 0, 0.1, 1, 10 or 100 ng LHRH/ml media for .75, 1.5, 3, 6, 12 or 24 hr. Values are means  $\pm$  SE.

tion that LHRH causes LH release from bovine pituitary cells in culture and that the amount of LH released is linearly related to the concentration of LHRH over the range 0.1 to 100 ng/ml (12). This result was demonstrable because the time of exposure to LHRH was as short as 15 min or as long as 24 hr. Additionally, the increase in LH release relative to basal was induced by each dose of LHRH was independent of time LHRH was present. This leads to the conclusion that the ability of LHRH to cause LH release appears to be consistent over at least 24 hr. Resolution of effects of estradiol on increasing LH concentration, as measured by difference in LH concentration between control cultures and those given estradiol, increased markedly with time. For this reason, time of exposure to LHRH of less than 3 hr may be desirable.

Our results confirm our previous observation that estradiol when present for 24 hr did not inhibit basal and LHRH induced LH release from bovine pituitary cells (12). These results provide evidence that estradiol is present for more than 3 hr before any inhibitory effects on LH release are demonstrated. Our failure to demonstrate an effect of estradiol at .75 or 3 hr agrees with results obtained using rat pituitary cells in culture in which a lag period may represent time required for estradiol to exert biological effects in gonadotrophs that result in inhibition of LH release. Inhibitors of protein synthesis block the stimulatory effect of low concentrations of estradiol on LH release (17). Failure to see an effect of estradiol during the first 3 hr of treatment may reflect time required for protein synthesis. Alternatively, this lag may be an artifact of the culture system.

#### I. EFFECT OF ESTRADIOL-17 $\beta$ AND TIME OF EXPOSURE TO ESTRADIOL ON MEDIUM LUTEINIZING HORMONE LEVELS.

Estradiol-17 $\beta$ (ng/ml)*			
0	5	50	Avg
8.6 $\pm$ .7 <sup>a</sup>	9.2 $\pm$ .6 <sup>a</sup>	9.2 $\pm$ .5 <sup>a</sup>	9.0
4.3 $\pm$ .9 <sup>a</sup>	21.3 $\pm$ .9 <sup>b</sup>	23.5 $\pm$ .9 <sup>b</sup>	16.4
2.5 $\pm$ 3.7 <sup>a</sup>	32.3 $\pm$ 3.6 <sup>b</sup>	41.7 $\pm$ 2.8 <sup>c</sup>	32.2
4.4 $\pm$ 2.4 <sup>a</sup>	80.7 $\pm$ 2.7 <sup>b</sup>	100.8 $\pm$ 4 <sup>c</sup>	82.0
27.5	35.9	43.8	

\* within time periods with different superscripts are significantly different at  $P < 0.05$ . Values are mean  $\pm$  SE ( $n = 12$ ).

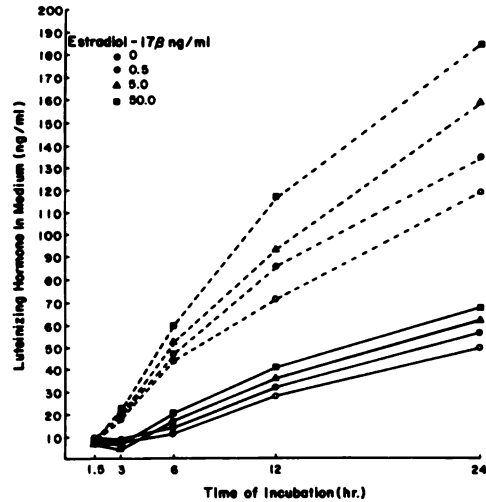


FIG. 2. LH concentration in medium of bovine pituitary cultures incubated with 0, .5, 5 or 50 ng/ml E<sub>2</sub> for 1.5, 3, 6, 12, or 24 hr with LHRH (0 or 100 ng/ml) present throughout the incubation period. Dashed lines represent data obtained when LHRH was present.

Considering that rate of accumulation of LH in medium accelerated during the 24 hr experimental period, failure to detect an effect of LH release at 45 min and 3 hr may be because LH release at this time is very low and gonadotrophs not receptive to this stimulus. An argument against the latter view is that LHRH was equally efficacious in causing LH release at all times tested. Our results also demonstrate that once estradiol affects LH release, this effect remains quantitatively similar at least to 24 hr in cultures incubated with and without LHRH. LH release by rat pituitary cells was increased by 500 ng/ml estradiol for 6 or 24 hr (15) or 0.27 ng/ml for 40 hr (11).

Results of experiments designed to investigate *in vivo* effects of estradiol on LHRH induced LH release revealed a biphasic effect i.e. estradiol first decreased, then increased magnitude of LHRH induced increase in serum (21-23). In these *in vitro* experiments, estradiol did not inhibit basal or LHRH induced LH release suggesting the initial inhibitory effect *in vivo* is not mediated via a direct effect on the pituitary.

**Summary.** Time course of 17- $\beta$  estradiol and luteinizing hormone-releasing hormone effect on LH release was studied using bovine pituitary cells on day 5 of culture. LHRH at concentrations of .1, 1, 10 and 100 ng/ml



increased LH in medium linearly with increasing log concentration of LHRH when present for .75, 1.5, 3, 6 and 24 hr and the percent increase over controls was same at each time period. In addition, estradiol (present for 6, 12 or 24 hr) at .5, 5, and 50 ng/ml also increased LH release linearly both in the presence or absence of LHRH. We conclude that the stimulatory effect of LHRH on LH release from bovine pituitary is consistent over 24 hr and the stimulatory effect of  $E_2$  on both basal and LHRH induced LH release may be mediated at least in part directly on the pituitary.

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## Effect of Administration of a LH-RH Inhibitory Analogue on Stages of the Rat Estrous Cycle<sup>1,2</sup> (40305)

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It has been demonstrated that synthetic analogues of LH-RH can block gonadotrophic surges of gonadotropins and inhibit estrus in hamsters (1) and rats (2, 3). The subcutaneous administration of 750 µg of [D-Leu<sup>6</sup>]-LH-RH four times on proestrus in hamsters, produced an 80% reduction of the LH surge and a 30% blockage of ovulation (1). In rats, 6 mg of [D-Phe<sup>2</sup>, D-Phe<sup>3</sup>]-LH-RH, injected in several doses on proestrus, brought about a inhibition of ovulation (2) whereas a dose of 1.5 mg of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH at noon on proestrus strongly inhibited LH and FSH surges and suppressed ovulation by 85% (3). Recently, Beattie *et al.* reported that [D-Phe<sup>2</sup>, D-Ala<sup>6</sup>]-LH-RH inhibited ovulation when it was given on days other than estrus in rats.

In *in vivo* assays, such as inhibition of induced LH release in immature rats, and blockade of ovulation in normal rats, [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH is more potent and longer acting than [D-Phe<sup>2</sup>, D-Phe<sup>3</sup>]-LH-RH (3, 5), which in turn is more potent than [D-Phe<sup>2</sup>, D-Ala<sup>6</sup>]-LH-RH (5). We therefore investigated the effects of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH on ovulation in rats injected at different stages of the estrous cycle or daily during estrus (E), diestrus 1 (D1) and diestrus 2 (D2).

**Animals and methods.** Adult female rats (River CD strain), weighing 200-250 g, were maintained under conditions of constant lighting (14 hr light and 10 hr dark-temperature (22°). Following a one

week period of adjustment to the animal house, their estrous cycles were determined by inspection of daily vaginal smears. Only those animals presenting at least two successive, regular 4-day cycles were used.

In the first experiment, the animals were injected s.c. with a single 1.5 mg dose of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH in 0.5 ml of vehicle or with vehicle alone (20% propylene glycol/saline solution) at noon of either E, D1, D2 or proestrus. Another group was injected at 9 AM on proestrus. On the following estrus, the animals were sacrificed and ovulation was checked by counting the number of ova under a dissecting microscope. The number of rats which ovulated compared to the total number of rats was considered an index of the antiovarian activity of the analogue.

In a second experiment, rats were injected s.c. with a 1.5 mg dose of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH twice a day (9 AM and 4 PM; total dose: 3 mg/day) during E, D1 and D2. No injection was given on proestrus. Control rats were injected with 0.5 ml of vehicle alone. On the following E, ovulation of both control and experimental animals was checked as described above. At 4 PM on each day of treatment, a blood sample from the jugular vein of control and experimental animals was collected within 20-30 sec under light ether anesthesia. The blood was centrifuged and sera separated and stored at -20° until assayed for LH and steroids. Some ovaries from control and [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH treated rats were removed at the time ovulation was being determined. The ovaries were fixed in Bouin's solution and then stained with hematoxylin-eosin (Bay Histology Service, San Rafael, CA). Vaginal smears were also examined daily during the period of treatment. Serum LH was determined by the double antibody radioimmunoassay method

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of Niswender *et al.* (6) as described elsewhere (7, 8). NIH-LH-S<sub>17</sub> was used as the standard preparation.

Estradiol and progesterone were measured in duplicate by the method of Abraham *et al.* (9) with slight modifications. About 1000 dpm of 2,4,6,7-<sup>3</sup>H-17 $\beta$  estradiol (SA:91, 3 Ci/mM) and of 1,2,6,7-<sup>3</sup>H progesterone (SA 103 Ci/mM) were added to one ml of plasma to estimate the recovery of the steroids. Each sample was extracted twice with anesthetic ether (Mallinckrodt). The ether extract was evaporated and the steroids were then resuspended in 1 ml of isooctane and were chromatographed on celite micro-columns. Progesterone was eluted with isooctane and estradiol with isooctane: ethyl acetate (3:2). The estradiol fraction was diluted in 0.5 ml of 0.1 M phosphate buffer (pH 7.8) containing 0.14 M NaCl, 0.01 M EDTA, 0.015 M sodium azide, and 0.1% gelatin. After an aliquot was taken to estimate steroid recovery, 0.2 ml of the solution was incubated with 2,4,6,7-<sup>3</sup>H-17 $\beta$  estradiol (0.1 ml/40,000 dpm) and with estradiol antiserum (0.1 ml at 1/100,000). The antiserum (S-310) was obtained from Abraham's laboratory. The estradiol recovery was 80%. The sensitivity of the assay was 2.5 pg/tube with an interassay coefficient of 8.5%.

The progesterone fraction was diluted with 1 ml of phosphate buffer; an aliquot was taken to estimate recovery and another (50

$\mu$ l) was incubated with 1,2,6,7-<sup>3</sup>H progesterone (0.1 ml/40,000 dpm) and with progesterone antiserum (0.1 ml at 1/7,000). The antiserum 3-oxime-BSA cross reacted with the following steroids: Testosterone and 20 $\alpha$ -OH-progesterone less than 1%, and 17 $\beta$ -OH-progesterone and deoxycorticosterone 2%. The recovery was 86%, the interassay coefficient of variation was 10%, and the sensitivity was 25 pg/tube. The free and bound hormones were separated using 0.2 ml of dextran-coated charcoal.

Duncan's new multiple range test (10) was used to analyze the significance of the differences in LH serum levels among the groups. The results from the ovulatory test were expressed as binomial data using one for ovulation and 0 for no ovulation; they were subjected first to analysis of variance (11, 12) and then compared by Duncan's new multiple range test (10) as described previously (5-8). The LH-RH analogue was prepared in our laboratory by the solid phase method (5). Its purity was confirmed by TLC and amino acid analysis.

**Results.** Table I shows the effect on ovulation of a single dose of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH injected at different days of the estrous cycle. It can be seen that when the analogue was injected at noon of proestrus, a 100% blockade of ovulation was observed. The degree of ovulation blockade decreased to 33% and 17% when the analogue was

TABLE I. BLOCKADE OF OVULATION IN THE RAT BY [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH (ANALOGUE) ADMINISTERED AT DIFFERENT STAGES OF THE ESTROUS CYCLE.<sup>a</sup>

Group	# of rats ovulated/total # of rats	% of blockade of ovulation	Mean $\pm$ SE of ova in ovulating rats
A. Proestrus (noon)			
1. Vehicle	4/4	0	12.2 $\pm$ 0.5
2. Analogue	0/6*	100	—
B. Proestrus (9 AM)			
3. Vehicle	4/4	0	13.0 $\pm$ 0.6
4. Analogue	4/6†	33.3	10.7 $\pm$ 0.9
C. Diestrus 2 (noon)			
5. Vehicle	4/4	0	12.7 $\pm$ 0.2
6. Analogue	5/6†	16.7	9.8 $\pm$ 1.1
D. Diestrus 1 (noon)			
7. Vehicle	4/4	0	12.0 $\pm$ 0.4
8. Analogue	6/6	0	11.6 $\pm$ 0.2
E. Estrus (noon)			
9. Vehicle	4/4	0	11.0 $\pm$ 0.4
10. Analogue	6/6	0	10.2 $\pm$ 0.6

<sup>a</sup> Dose of analogue: 1.5 mg/rat at the time shown in parenthesis. Duncan's new multiple range test: \* Significantly different from the respective control value. † Significantly different from the value of Group 2.

and at 9 AM on proestrus and on D2, respectively. Ovulation was not blocked after giving the analogue on D1 and the previous evening (Table I).

The effect of daily injections for three days (E, D1, and D2) of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH is presented in Table II. Two out of five rats treated with the analogue ovulated (86% blockade of ovulation), one of them fully (12 ova) and the other partially (4 ova). On the other hand, only one of the five control rats failed to ovulate (Table II).

Figure 1 shows the effect of daily injections of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH on the LH levels. In the analogue-treated rats, the LH level was significantly lower ( $P < 0.01$ ) than the control group, when they were compared on the evening of proestrus. This demonstrates that the injection of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH during E, D1 and D2 inhibited the LH surge that was seen in the control animals on the afternoon and evening of proestrus.

Figure 2 shows the effects of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH on serum levels of steroids. Estradiol in the treated rats was not significantly different from the control group. However, progesterone was higher on D1 and on the afternoon of proestrus in those animals treated with the analogue. Moreover, the animals treated with the analogue showed a normal vaginal smear on the afternoon of proestrus.

Ovaries of the anovulatory animals treated with [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH showed a histological pattern similar to ovulated untreated animals; normal follicular development including antral follicles were present, although corpora lutea hemorrhagica were absent in the latter animals.

**Discussion.** Beattie *et al.* (4) reported that [D-Phe<sup>2</sup>, D-Ala<sup>6</sup>]-LH-RH significantly inhibited ovulation when it was injected on

TABLE II. BLOCKADE OF OVULATION IN THE RAT BY [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH (ANALOGUE) ADMINISTERED ON E, D1 AND D2.<sup>a</sup>

Group	# of rats ovulated/total # of rats	% of blockade of ovulation	Mean $\pm$ SE of ova in ovulating rats
Control	13/14	7.1	11.8 $\pm$ 0.3
Analogue	2/14	85.7*	8.0 $\pm$ 4.0 <sup>b</sup>

<sup>a</sup> Dose of analogue: 1.5 mg/rat twice a day (total dose: 3 mg/day). <sup>b</sup> One rat ovulated four ova. Duncan's multiple range test: \* Significantly different from the control group.

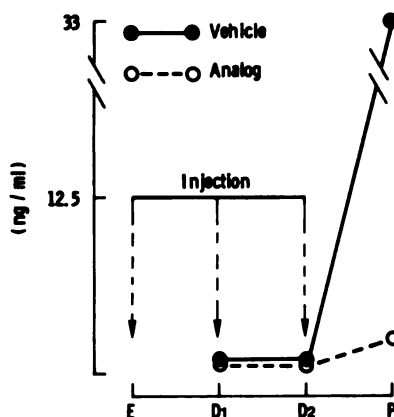


FIG. 1. Effect of the administration of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH on serum LH levels. Animals were injected s.c. with either the analogue (1.5 mg) or vehicle at 9 AM and 4 PM on E, D1 and D2. Blood was taken at 4 PM. Each point represents the mean  $\pm$  SE of six rats.

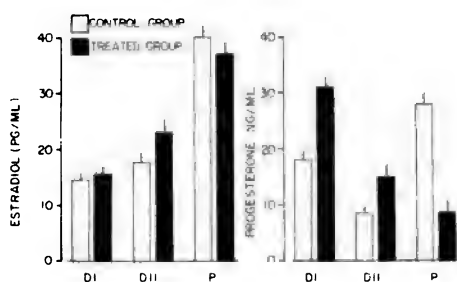


FIG. 2. Effect of administration of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH on serum steroid levels. Animals were injected s.c. with either the analogue (1.5 mg) or vehicle at 9 AM and 4 PM on E, D1 and D2. Blood was taken at 4 PM. Each point represents the mean  $\pm$  SE of six rats.

different days of the estrous cycle in 4-day cycling rats. Ovulation was inhibited by 97%, 87% and 79% after proestrus, D2 and D1 injection of the analogue, respectively. Using a more potent analogue, [D-Phe<sup>3</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH (5), we were able to inhibit ovulation considerably when this analogue was injected either on the morning or at noon of proestrus. Only a 17% inhibition of ovulation was observed when [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH was injected at noon on D2 and no inhibition of ovulation was seen when it was injected on the previous E. Apparently, the length of the action of [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH is not sufficient to block ovulation when it is injected before D1. Furthermore, the injection of 1.5 mg twice a day at 9 AM and 4 PM (total dose of 3 mg/rat/day)

on E, D1 and D2, brought about almost complete suppression of the LH surge on the next day (Proestrus) and an 86% blockade of ovulation on the following estrus morning, without altering the normal vaginal smear pattern. This might be due to unaltered serum levels of estradiol after the treatment (Fig. 2). Discrepancies between our results and those obtained by Beattie *et al.* (4) might be due to the different schedule of treatment and doses used.

It is interesting to point out the changes in serum progesterone levels observed in the animals treated with the analogue on E, D1 and D2 throughout the experiment. They were higher on D1 and lower on proestrus when they were compared with those of the control rats. Because the peak of serum LH levels in the rats of our colony occurs between 3 and 4 PM, the low proestrous afternoon levels of progesterone could be due to a blockade of LH release produced by direct effects of the analogue on the pituitary and hypothalamus. The lack of LH release could have impaired the subsequent ovulation and luteinization. On the other hand, high progesterone levels during D1 might have contributed to the blockade of the LH surge and ovulation. It has been demonstrated that administration of progesterone or synthetic analogues early in the cycle depresses proestrus serum LH and FSH and delays ovulation (13-17).

In conclusion, using antagonist analogues of LH-RH it is possible to block ovulation without affecting the rat estrous cycle. Thus, the possibility exists to develop an even more potent analogue which can be used in humans without altering plasma estrogen levels.

**Summary.** [D-Phe<sup>2</sup>, Phe<sup>3</sup>, D-Phe<sup>6</sup>]-LH-RH, a potent antagonist of LH-RH, was injected during the different stages of the estrous cycle in rats at a dose of 1.5 mg/rat. When it was administered at noon or proestrus, a 100% blockade was observed. This decreased to 33% and 17% when the analogue was injected at 9 AM on proestrus and diestrus 2, respectively. No blockade of ovulation was observed after the injection of the analogue on diestrus 1 or on the previous estrus. The potential administration of the analogue daily on E, D1 and D2, brought about complete suppression of LH surge on

proestrus and an 86% blockade of ovulation without altering the cyclic vaginal smear. In this case, serum levels of estradiol were not modified but progesterone levels were significantly lower on proestrus higher on diestrus 1 in the analogue group as compared to control rat higher level of progesterone on diestrus 1 might account in part for the inhibitory LH-surge and blockade of ovulation inhibitory analogues of LH-RH.

We thank Mrs. J. Gauthier and Mrs. J. L. their valuable technical assistance; Dr. G. N. Dr. Ward and NIAMDD-Rat-Pituitary Hormone Program for the gifts of materials used in radioassays.

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## ANNUAL REPORT

### Annual Report of the Secretary-Assistant Treasurer and Managing Editor for the Year Ending December 31, 1977

*Finance.* The following is an abbreviated financial report prepared by Leo Kaden, C.P.A., of Padell, Kaden, Zell and Co.

Balance of cash in banks at January 1, 1977	\$29,153
Receipts:	
From "Proceedings":	
Subscriptions	\$155,973
Advertising	7,298
Publication charges	37,778
Friends	46,383
Royalty	8
Other receivables	161
Total from "Proceedings"	<u>252,901</u>
Dues	58,518
Manuscript	7,562
Excess of investments sold over investments purchased	352
Dividends interest and income	17,721
Sundry receivables	2,286
Monies received from Internal Revenue Service	5,000
Due from Michigan Section	632
Contributions	54
Refunds of Honorariums	362
Total receipts for period	<u>345,388</u>
Total funds available	<u>374,541</u>
Disbursements:	
From "Proceedings":	
Academic Press	249,078
Printing	2,042
Total for "Proceedings"	<u>251,120</u>
Office supplies, postage, and telephone	10,386
Subscriptions, refunds, and Honorarium	2,650
Meetings and travel	3,862
Pension	4,519
Salaries and payroll taxes	41,048
Professional fees	1,250
Bank charges and miscellaneous expenses	150
National Society for Medical Research	1,500
Total disbursements for the period	<u>316,484</u>
Balance of cash in bank at December 31, 1977	<u>\$58,056</u>

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*Editors.* The Editorial and Publication Committee consists of: Drs. M. Zucker, Chairperson; I. Clark, M. Hilleman, S. I. Morse, and S. Seifter.

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*Election.* The mail ballot has resulted in the election of Dr. R. W. Berliner, as President-Elect for 2 years beginning January 1, 1978; Dr. G. W. Siskind, Treasurer, and Dr. M. R. Nocenti, Secretary-Assistant Treasurer, for a similar period.

The following were elected members of the Council for a period of 4 years: Drs. A. H. Briggs, H. F. DeLuca, J. P. Gilmore, M. W. Orsini, E. E. Selkurt, and D. B. Zilversmit.

*Tellers.* Drs. M. Blank and R. Emmers.

Miss Felice M. O'Grady has been in charge of the duties of the Society's National Office.

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1977

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm <sup>3</sup>	molal (concentration)	<i>m</i>
Curie	Ci	molar (concentration)	<i>M</i>
degree Celsius (Centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	<i>N</i>
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	μl	square centimeter	cm <sup>2</sup>
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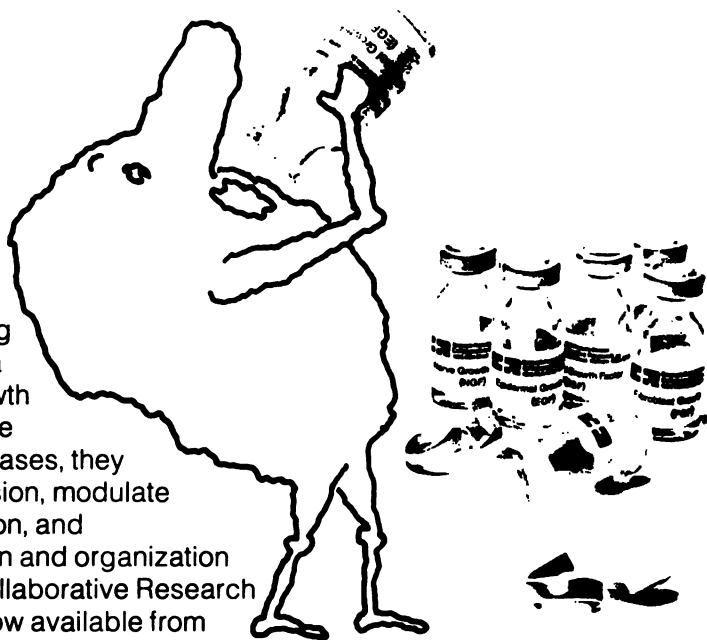
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# Effect of Renal Prostaglandin Synthesis and Metabolism by Indomethacin in Rats (40306)

HARD J. ROMAN,<sup>1</sup> MICHAEL L. KAUKER, NORBERTO A. TERRAGNO,  
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Indomethacin, because of its potency as an inhibitor of prostaglandin biosynthesis *in vivo*, is widely employed as a pharmacological agent to investigate the renal actions of endogenous prostaglandins. Evidence for inhibition of renal prostaglandin production *in vivo* has usually been established by demonstrating a reduction of prostaglandin release, lowering of prostaglandin (PG) concentration in renal venous blood or a decreased urinary excretion of prostaglandins. However, there are few studies in which the ability of indomethacin to inhibit renal prostaglandin synthesis has been systematically evaluated and none of the studies has been done in surgically prepared rats for acute experimentation. Furthermore, release of prostaglandins by the kidney probably reflects the activity of enzymes that synthesize and release prostaglandins. Assessment of prostaglandin release may not be adequate to determine the extent of inhibition of prostaglandin synthesis by indomethacin *in vivo*. Indomethacin has been reported to inhibit *in vitro* the primary prostaglandin catalyzing enzymes: 15-hydroxyprostaglandin synthase (PGDH) and prostaglandin G/H oxidoreductase (9-KRD). Indeed, Teret et al. (2) have recently shown that indomethacin does not inhibit renal release of prostaglandin E<sub>2</sub> in conscious dogs. In the present study, the effect of indomethacin (2 mg/kg) on prostaglandin release, synthesis and metabolism was investigated in anesthetized non-diuretic rats.

**Materials and methods.** Male Wistar rats weighing between 200-400 g were anesthe-

tized with ip Inactin, 100 mg/kg of body wt. After tracheostomy, cannulas were placed in the right external jugular vein for infusions and the right carotid artery for recording of blood pressure. The left kidney was exposed and a polyethylene cannula (PE-50) was placed in the left ureter to allow for urine collections (3). The following drugs were used in the present study: indomethacin (Merck, Sharp and Dohme), meclofenamate (Parke Davis & Co.), phenylbutazone (Geigy Co.), RO 20-5720 (Hoffman La Roche, Inc.). The following three types of studies were carried out.

(a) *Prostaglandin bioassay.* In each experiment, two rats were surgically prepared as described above and, after one hour equilibration, both members of the pair received either indomethacin (2 mg/kg), meclofenamate (2 mg/kg), RO 20-5702 (2 mg/kg), phenylbutazone (50 mg/kg) or 3 mM sodium carbonate vehicle alone. Drugs were infused iv at a rate of 40  $\mu$ l/min in an approximate total volume of 0.2 ml/100 g body wt. After 30 min, a 5 ml blood sample was collected from the left renal vein over a 1 to 2 min period. Blood samples from the two rats were pooled and injected into ice-cold ethanol. Samples were bioassayed for prostaglandin E<sub>2</sub>-like activity after an acidic lipid extraction as described previously (4). Since the extracts of blood samples were not chromatographed to separate the various prostaglandins, the reported values represent total prostaglandins and are expressed as the concentration of PGE<sub>2</sub>-like substance in the original samples without correction for losses (10-15%) that occur during the extraction procedure (4).

(b) *Prostaglandin synthesis.* In each of these experiments, two rats were prepared as above. After a 1 hr equilibration, urine flow and blood pressure were recorded during two clearance periods of 10 min each. The rats were then infused with either indomethacin

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(2 mg/kg, 4 experiments) or vehicle (3 experiments) as described above. Urine flow and blood pressure were again measured during two experimental clearance periods after a 30-min equilibration. The kidneys from the two rats were removed and the renal papillae were quickly excised and homogenized in ice-cold 0.05 M  $\text{KH}_2\text{PO}_4$  buffer, pH 7.4, with a Polytron homogenizer. Aliquots of papillary homogenates equivalent to 50 mg of wet tissue were incubated at 37° for 30 min in 2 ml of 0.05 M  $\text{KH}_2\text{PO}_4$  buffer containing 0.4  $\mu\text{Ci}$  of 1-[ $^{14}\text{C}$ ]arachidonic acid and 2 mM reduced glutathione (5). The reaction was stopped by acidification with 1 M citric acid (final pH 3.0). The reaction mixture was extracted 3 times with 6 ml of ethylacetate. The combined extract was evaporated under nitrogen. The resulting residue was dissolved in 100  $\mu\text{l}$  of chloroform: methanol (1:1, v/v), quantitatively spotted on thin-layer chromatographic plates, and separated by chloroform:methanol:acetic acid:water (90:9:1:0.65, v/v) as the solvent system. Assays were run in duplicate. Prostaglandin production in boiled tissue controls was subtracted to correct for nonenzymatic formation (5).

(c) *Prostaglandin metabolism*. Eight additional rats were prepared and infused with indomethacin or vehicle as in the prostaglandin synthesis studies. In each experiment, the kidneys were removed after the experimental clearance periods, the renal cortex and outer medulla were excised and homogenized as described. The soluble enzyme fractions containing the PG metabolic enzymes were obtained by high speed centrifugation (105,000g). The fractions thus acquired were used to determine the effect of indomethacin on the activity of 9-KRD and PGDH (both  $\text{NAD}^+$  and  $\text{NADP}^+$  dependent) using procedures described previously (6, 7). In brief, PGDH activity was assayed by incubating aliquots of the high speed supernatant at 37° for 10 min with  $\text{NAD}^+$  or  $\text{NADP}^+$  (4 mM), 3H-PGE<sub>2</sub> (0.56  $\mu\text{M}$ ; 300,000 dpm, NEN, Boston, MA) and 0.05 M  $\text{KH}_2\text{PO}_4$  buffer, pH 7.4, in a final volume of 1 ml. The reaction was stopped by acidification with 1 M citric acid to pH 3.0. Authentic PGE<sub>2</sub> and 15-keto PGE<sub>2</sub> standards were added to the assay mixture and extracted 3 times with 2 ml of ethylacetate. The extract was dried under a stream

of nitrogen. The residue was redissolved in 100  $\mu\text{l}$  of chloroform:methanol (1:1, v/v). An aliquot of 50  $\mu\text{l}$  of the extract was applied to a thin-layer chromatographic plate, 0.25 mm thick, 20 × 10 cm, silica precoated plastic sheets, Brinkman, NY, separated in iso-octane: ethyl acetate: acid:water (25:55:10:50, v/v). PGE<sub>2</sub> and 15-keto metabolite were located by exposing the TLC plate to iodine vapor, followed by spraying the plate with 10% phosphoribidic acid in ethanol. Areas corresponding to authentic PGE<sub>2</sub> and 15-keto PGE<sub>2</sub> standards were cut out and suspended in 100  $\mu\text{l}$  of 0.4% Omnifluor toluene liquid scintillation fluid and counted in a Nuclear Chicago II liquid scintillation spectrometer. Total counts were converted to dpm using a quench correction curve and external standard channel ratios. The results are expressed as pmoles of 15-keto PG formed per nmol of protein.

9-KRD activity was determined in the presence of an NADPH generating system (7) containing: NADPH, 0.15 mM, glucose 6-phosphate, 3.5 mM; 2 units of glucose 6-phosphate dehydrogenase; 3H-PGE<sub>2</sub> and 0.05 M  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4), and the enzyme fraction in a final volume of 1 ml. After 10 min incubation at 37°, the reaction was stopped by acidification with 1 M citric acid to pH 3.0. Samples were extracted and separated by thin-layer chromatography as described above. Areas corresponding to authentic PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  standards were cut out and the radioactivity was determined as before. Protein concentration was determined by the method of Lowry *et al.* (8) using serum albumin as standard. All assays were carried out in triplicate and controls were run simultaneously using boiled supernatant. Results are presented as the mean  $\pm$  SEM. Significance was determined by Student's *t*-test.  $P < 0.05$  was considered significant.

In order to establish the relationship between different *in vitro* doses of indomethacin and inhibition of renal cortical enzyme activity, the effect of increasing concentrations of indomethacin (0–50  $\mu\text{g}/\text{ml}$ ) on three major renal metabolic enzymes were investigated. Assay procedures were similar to those described above, different concentrations of indomethacin were added to the incubation

indicated (Fig. 1).

**Results.** Urine flow and blood pressure were measured in these studies to obtain an indication of the physiologic state of the rats under the experimental conditions. Control urine flows were similar in both vehicle and indomethacin treated rats, averaging  $1.70 \pm 0.26$  and  $2.07 \pm 0.51$   $\mu\text{l}/\text{min}/100$  g b wt respectively. After indomethacin urine flow decreased 41% ( $P < 0.05$ ), whereas after infusion of an equal volume of vehicle alone it increased 61% ( $P < 0.05$ ). Mean systemic blood pressure was unchanged after administration of indomethacin (from  $116 \pm 4$  to  $113 \pm 4$  mm Hg,  $P > 0.1$ ) or vehicle (from  $124 \pm 4$  to  $123 \pm 3$  mm Hg,  $P > 0.2$ ).

The concentration of prostaglandin  $E_2$ -like substance in renal venous blood of vehicle pretreated rats (Table I) was approximately 17-fold greater than levels measured in arterial blood of two additional pairs of animals ( $66 \pm 6$  pg/ml,  $P < .01$ ), indicating that prostaglandin found in the venous blood of these rat kidneys was of renal origin. Mean renal venous blood prostaglandin levels were significantly lowered, by 69% and 90%, respectively, in rats infused with indomethacin or meclofenamate. Similarly, in single experiments 2 other nonsteroidal anti-inflammatory drugs (NSAID), phenylbutazone and

RO 20-5702 appeared to reduce renal prostaglandin release (Table I).

The effect of indomethacin pretreatment on prostaglandin synthetase activity of renal papillary homogenates was also studied *in vitro*. Pretreatment with indomethacin, 2 mg/kg, significantly reduced the synthesis of prostaglandins  $E_2$ ,  $D_2$  and  $F_{2\alpha}$  from their precursor arachidonic acid (Table II). Renal papillary  $\text{PGE}_2$  production was inhibited 97% by *in vivo* indomethacin pretreatment. Addition of indomethacin, 5  $\mu\text{g}/\text{ml}$ , to incubations of renal papillary homogenates obtained from vehicle pretreated rats also diminished prostaglandin production. The degree of prostaglandin synthetase inhibition produced by addition of indomethacin *in vitro* (5  $\mu\text{g}/\text{ml}$ ) and pretreatment with indomethacin *in vivo* (2 mg/kg) was similar.

Indomethacin pretreatment also interfered with renal prostaglandin metabolism in the present studies. The effect of indomethacin on the key prostaglandin metabolic enzymes is shown in Table III. Treatment with indomethacin inhibited renal cortical-medullary 9-KRD activity by 61% ( $P < 0.05$ ).  $\text{NAD}^+$ -dependent PGDH activity was also diminished by 46%, however this decrease was not statistically significant. The enzyme  $\text{NADP}^+$ -dependent PGDH was not affected by indomethacin.

The effect of indomethacin pretreatment

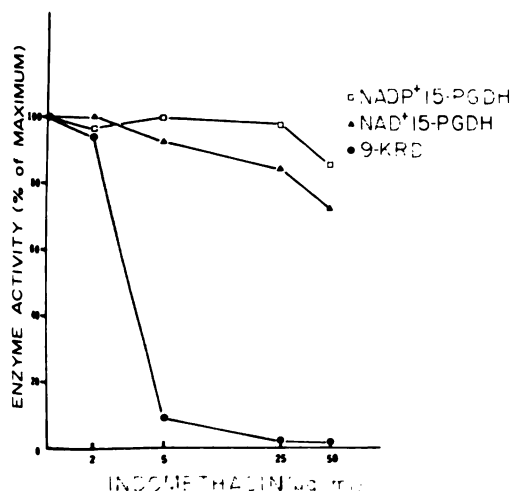


FIG. 1. Dose-response relationship of indomethacin on renal cortical prostaglandin metabolic enzyme activities *in vitro*. The effect of indomethacin was expressed as per cent of enzyme activity after correction for the control. Each point represents the mean of duplicate determinations; controls were run without indomethacin.

TABLE I. EFFECT OF INDOMETHACIN AND OTHER NSAID<sup>a</sup> ON THE CONCENTRATION OF PROSTAGLANDIN  $E$ -LIKE SUBSTANCE IN RENAL VENOUS BLOOD OF RATS.

	Prostaglandin concentration <sup>b</sup> pg/ml $\text{PGE}_2$ -like equivalents
Vehicle (7)	$1102 \pm 167$
Indomethacin, 2 mg/kg (6) <sup>c</sup>	$343 \pm 87^c$
Meclofenamate, 2 mg/kg (3)	$108 \pm 17^d$
Phenylbutazone, 50 mg/kg (1)	129
RO 20-5702, 2 mg/kg (1)	496

Mean values  $\pm$  SE are presented.

<sup>a</sup> NSAID = nonsteroidal anti-inflammatory drugs.

<sup>b</sup>  $\text{PGE}_2$ -like material was assayed on a cascade of rat stomach strip, rat colon and chick rectum.

<sup>c</sup>  $P < 0.005$ , statistically different from vehicle treated animals.

<sup>d</sup>  $P < 0.001$ , statistically different from vehicle treated animals.

<sup>e</sup> Numbers in parentheses = number of samples assayed. Each sample contained two 5 ml samples of renal venous blood obtained from two rats.

TABLE II. PROSTAGLANDIN BIOSYNTHESIS BY HOMOGENATES OF RENAL PAPILLAE FROM RATS PRETREATED WITH VEHICLE OR INDOMETHACIN.

	Rate of prostaglandin biosynthesis <sup>a</sup>			
	PGE <sub>2</sub>	PGF <sub>2α</sub>	PGD <sub>2</sub>	Total PG
Vehicle pretreated (3) <sup>d</sup>	2.65 ± 0.48	0.68 ± 0.20	0.17 ± 0.02	3.49 ± 0.48
Indomethacin pretreated (2 mg/kg) (4)	0.08 ± 0.04 <sup>c</sup>	0.06 ± 0.01 <sup>b</sup>	0.02 ± 0.01 <sup>c</sup>	0.16 ± 0.01 <sup>c</sup>
Indomethacin added <i>in vitro</i> (5 µg/ml) (4)	0.18 ± 0.08 <sup>c</sup>	0.15 ± 0.12	0.05 ± 0.03 <sup>b</sup>	0.38 ± 0.18 <sup>c</sup>

Mean data ± SE are presented.

<sup>a</sup> Values expressed as picomoles of prostaglandin formed/min per mg wet wt of tissue.

<sup>b</sup> *P* < 0.05, compared to vehicle pretreated.

<sup>c</sup> *P* < 0.005, compared to vehicle pretreated.

<sup>d</sup> Numbers in parentheses = number of experiments.

on the PG metabolic enzymes were also confirmed by the *in vitro* experiments. Indomethacin at a dose of 5 µg/ml *in vitro* produced marked inhibition of PG 9-KRD but was less effective on NAD<sup>+</sup>-dependent PGDH. At a dose of 25 µg/ml 9-KRD was inhibited 95% while NAD<sup>+</sup>-dependent PGDH activity was lowered only 15%. However, at this dose range indomethacin produced little or no effect on NADP<sup>+</sup>-dependent PGDH (Fig. 1).

**Discussion.** In the present investigation, inhibition of renal prostaglandin synthetase after administration of 2 mg/kg indomethacin to anesthetized nondiuretic rats was assessed by two methods. These experiments demonstrated the following: (a) the concentration of a PGE-like substance in the renal venous blood was reduced 69% by indomethacin; (b) indomethacin pretreatment decreased, by greater than 90%, the conversion of radiolabeled arachidonic acid to various prostaglandins (PGE<sub>2</sub>, F<sub>2α</sub> and D<sub>2</sub>) by renal papillary homogenates; (c) NSAID other than indomethacin were also effective in lowering renal venous prostaglandin levels. Indomethacin *in vivo* reduced, but did not com-

pletely abolish, net renal prostaglandin output in anesthetized rats prepared for acute experimentation. Associated with an inhibition of prostaglandin production was a significant decline in urine flow, which is consistent with the proposal that prostaglandins affect tubular handling of water by attenuating the antidiuretic action of ADH (9).

The extent of renal prostaglandin synthetase inhibition by indomethacin, as determined by the decline in renal venous prostaglandin levels (69%), was lower than that estimated by *in vitro* prostaglandin production by papillary homogenates (97%). The dissimilar degree of inhibition indicated by the two methods may reflect inherent differences in the experimental procedures. Homogenization of the renal papillae in the tissue incubation studies, for example, may have allowed indomethacin greater access to the enzyme cyclo-oxygenase thus producing a more complete blockade of prostaglandin synthesis than that which occurred *in vivo*. On the other hand, the present studies provide evidence suggesting an alternative explanation; i.e., the degree of prostaglandin syn-

TABLE III. METABOLISM OF PROSTAGLANDIN E<sub>2</sub> BY THE SOLUBLE ENZYME FRACTION OF RENAL CORTEX AND OUTER MEDULLA FROM RATS PRETREATED WITH VEHICLE OR INDOMETHACIN.

	15-PGDH <sup>a</sup>		PG-9-KRD <sup>b</sup>
	NAD <sup>+</sup> dependent	NADP <sup>+</sup> dependent	NADPH dependent
Vehicle pretreated (4) <sup>c</sup>	2.12 ± 0.66	0.98 ± 0.12	0.88 ± 0.18
Indomethacin pretreated, 2 mg/kg (4)	1.14 ± 0.31 <sup>c</sup>	1.04 ± 0.12 <sup>c</sup>	0.34 ± 0.12 <sup>d</sup>

Mean data ± SE are presented.

Values are expressed as picomoles of PGF<sub>2α</sub> or 15-keto PGE<sub>2</sub> formed/min per mg protein.

<sup>a</sup> 15-PGDH = 15-hydroxyprostaglandin dehydrogenase activity.

<sup>b</sup> PG-9-KRD = Prostaglandin E<sub>2</sub> 9-ketoreductase activity.

<sup>c</sup> Not significant *P* > 0.05.

<sup>d</sup> *P* < 0.05 compared to vehicle pretreated.

<sup>e</sup> Numbers in parentheses = number of experiments.

thesis inhibition after indomethacin may not have been accurately reflected by the decline in renal prostaglandin release because the drug impaired prostaglandin metabolism as well as synthesis. Such a conclusion is supported by our finding that 9-KRD activity of renal cortico-medullary homogenates was reduced by 61% after indomethacin pretreatment. Additionally, although a significant difference was not detected in the present prostaglandin metabolism study, the decline of 46% in mean  $\text{NAD}^+$ -dependent PGDH activity after indomethacin is consistent with the view that indomethacin affects both prostaglandin synthesis and metabolism. The finding that indomethacin inhibited the soluble enzyme, 9-KRD, after systemic administration implies that this compound gained access to sites located in the intracellular compartment.

Inhibition of renal cyclo-oxygenase, 9-KRD and PGDH by indomethacin and other NSAID *in vitro* has been reported previously (10, 11). The concentrations used for half-maximal inhibition of PG synthesis were of the same order of magnitude as the concentration shown to produce half-maximal inhibition of prostaglandin metabolic enzymes. The present observations, however, provide the first evidence that a standard *in vivo* dose of indomethacin, 2 mg/kg, producing an estimated unbound plasma concentration of 5  $\mu\text{g}/\text{ml}$ , interferes with prostaglandin metabolism. The effect on the PG metabolic enzymes was confirmed by the *in vitro* experiments which indicated that indomethacin indeed affected the major metabolic route of PGs in the kidney. The additional observation, both *in vitro* and *in vivo*, that 9-KRD activity was markedly reduced by NSAID especially by indomethacin whereas the enzyme  $\text{NADP}^+$ -dependent PGDH was unaffected, suggests that these enzymes may have different active site(s) even though they have been reported to be identical (12).

In conclusion, indomethacin, meclofenamate and other NSAID markedly reduced net renal prostaglandin production in rats surgically prepared for acute experimentation. It appears from the data reported here that indomethacin, after *in vivo* administration, may have a complex action to impair both synthesis and metabolism of renal pros-

taglandins. Differential inhibition of the enzymes involved in net prostaglandin production and alterations in the types of prostaglandins formed in various parts of the kidney complicate the interpretation of data obtained during indomethacin treatment. The usefulness of this agent to evaluate the role of prostaglandins in the regulation of renal function may thus be limited. However, due to species differences which exist with respect to prostaglandin degradation, the conclusion of this study may not be extrapolated to other species.

**Summary.** The effect of indomethacin and other NSAID on renal prostaglandin synthesis and metabolism was studied in nondiuretic rats prepared for acute experimentation. Thirty minutes after the administration of a 2 mg/kg iv dose of indomethacin, the concentration of prostaglandin in renal venous blood as determined by bioassay was reduced 69%. In addition, conversion of radiolabeled arachidonic acid to prostaglandin  $\text{E}_2$  *in vitro* by the renal papillae of indomethacin pretreated rats was inhibited 97%.

Pretreatment with indomethacin also inhibited renal cortical-medullary prostaglandin  $\text{E}_2$  9-ketoreductase activity by 61%.  $\text{NAD}^+$ -dependent 15-hydroxy-prostaglandin dehydrogenase activity was diminished 46%; however, this inhibition was not statistically significant.  $\text{NADP}^+$ -dependent 15-hydroxy-prostaglandin dehydrogenase activity was unaffected by pretreatment. It is concluded that indomethacin alters net renal prostaglandin production by inhibiting both prostaglandin synthesis and its metabolism.

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## Magnesium Deficiency on Intestinal Calcium Transport in Rats<sup>1, 2</sup> (40307)

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Ca absorption in rats has been increased (1-4), unaffected (5, 6), or decreased (7) as a result of magnesium deficiency. Possible factors accounting for these differences are the degree of magnesium deficiency, length of the depletion period, and effects of inanition or alterations in the diet of the deficient animals. Another factor that makes it difficult to compare findings from different laboratories is the technique used to determine Ca absorption. In the exception of two studies (1, 2), Ca absorption was measured by the *in situ* perfusion method, previous investigations of Ca transport in magnesium deficiency were carried out by means of *in vivo* techniques (3-7). To obtain results somewhat more applicable to the magnesium depletion in the intact rat, avoiding the inaccuracies of the *in situ* perfusion procedure, we used the ligated loop perfusion techniques to estimate Ca transport across the small intestine. Both techniques were carried out *in vivo* and permit measurement of Ca fluxes in a defined intestinal segment. *In situ* perfusion has the advantage of allowing estimation of Ca steady state, using serial perfusions of the same animal with solutions of varying concentrations.

**and methods.** Male Sprague-Dawley rats weighing 120-130 g, were pair-housed which lasted for either 14 or 28 days on the magnesium deficient and control diets were made from a basal diet previously described in detail elsewhere (8). Quantities of magnesium sulfate were added to the basal diet to final concentrations of 10 ppm  $Mg^{2+}$  for the deficient diet and 100 ppm  $Mg^{2+}$  for the control diet. The

diets contained 20% casein, 0.6% Ca and 0.4% P. Deionized water was allowed *ad libitum*.

**Experiment 1.** The rate of Ca absorption across the duodenum and ileum was determined in rats depleted of magnesium for 14 and 28 days. All transport estimates were carried out after an overnight fast. Ca transport was determined by the *in vivo* ligated loop technique described by Wasserman and Taylor (9). Ten centimeters of duodenum and 15 cm ileum were ligated in each rat. Each loop was injected with 0.5 ml of a dosing solution containing 0.1  $\mu Ci$   $^{47}Ca$  (Cambridge Nuclear, Billerica, MA) in 5 mM  $CaCl_2$  and 150 mM NaCl, pH 7.2. Five, 15, 30, 60, or 90 min after dose injection into the loop, the rats were anaesthetized with ether and the ligated loops were excised. Blood samples, obtained by cardiac puncture, were collected in tubes containing  $Na_2EDTA$ . The left tibiae were removed and cleaned of connective tissue and muscle. Radioactivity measurements were made in all intestinal loops and cleaned bones by use of a Nuclear Chicago well-type NaI crystal automatic gamma counter. Ca absorption was calculated by subtracting percent injected  $^{47}Ca$  remaining in the loop from 100%. The difference was designated %  $^{47}Ca$  transferred to the body.

**Experiment 2.** The *in situ* perfusion technique was used in rats magnesium depleted for 14 days to study Ca fluxes in the duodenum. The procedure has been described in detail by Wasserman *et al.* (10). Each rat was perfused in sequence with five dosing solutions, each containing 150 mM NaCl, 0.1-0.4  $\mu Ci$   $^{47}Ca/ml$ , and 0.5, 1.0, 2.0, 10.0 or 20.0 mM Ca. All solutions were adjusted to pH 7.2-7.4. The solutions were perfused into the ligated duodenal loop in order of increasing Ca concentration at a rate of 4-5 ml/hr using a motorized syringe pump (Harvard Apparatus Co., Dover, MA). An initial equilibration period of 40 minutes was allowed for each concentration of Ca perfused, and the outflow solution collected during this period

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<sup>2</sup>Presented here were submitted in partial fulfillment of the requirements for the Ph.D. degree.

was discarded. During the subsequent perfusion, carried out for 30 min, the outflow solutions were collected in graduated tubes. Volumes were measured to obtain estimates of water inflow and outflow rates. Ca influx was calculated as follows (10): Ca influx (lumen to blood) =  $(^{47}\text{Ca}_i)(W_i) - (^{47}\text{Ca}_o)(W_o)/[(\text{SA}_i + \text{SA}_o)/2] \times L$  where:  $^{47}\text{Ca}$  = radiocalcium content of fluids (cpm/ml); SA = specific activity of fluid Ca (cpm/mole); W = rate of water flow (ml/hr); L = length of duodenal segment (cm); i, o = superscripts referring to inflowing and outflowing solutions respectively.

Previous studies showed that calcium is absorbed by two processes, one process is saturable and the other has the characteristics of diffusion (11). In the present series, the rates of passive and saturable diffusion were calculated as follows. A straight line parallel to the diffusional component of each influx curve was drawn through the origin. At 0.5, 1, 2, 10 and 20 mM Ca concentrations the values for passive diffusion were subtracted from the corresponding values for Ca influx. The differences, representing the portion of influx due to saturable transport, were plotted against luminal Ca concentration.

**Ca and Mg content of blood and tibia.** The tibiae were dried at 90° for 3–4 days and ashed at 550° for 16–20 hr in a Thermolyne muffle furnace. The ash was dissolved in 3 ml concentrated HCl. Suitable dilutions of tibia ash or of plasma were analysed for Mg

and Ca by atomic absorption using a P Elmer atomic absorption spectrophotometer Model 290 B.

**Statistical analysis.** All analyses were using the paired *t* test. Levels of significance were based on the differences between of Mg deficient and control rats (13).

**Results. Response to magnesium depletion.** Weight gains were similar in magnesium deficient and control rats for the initial 10 days of Mg depletion. By day 14, however, body weights of the magnesium deficient rats were significantly below those of their fed controls. By this time plasma Mg concentrations were markedly reduced and remained at this low level throughout the days of depletion (Table I). Plasma Ca showed some variability. Mean values significantly elevated in the rats Mg deficient for 14 days in experiment 2, when there were seven animals per group. No statistically significant differences were seen in rats deficient for 14 or 28 days in experiment 1 when there were only three rats per group. The magnesium content of the tibiae was significantly reduced and the calcium content slightly significantly increased after 28 days depletion. (Table II).

**Intestinal Ca transport.** Intestinal Ca transport (%  $^{47}\text{Ca}$  transferred from the lumen to the blood) was consistently less in the magnesium deficient rats than in their paired controls. The difference between the two groups was significant after 2 weeks of

TABLE I. BODY WEIGHTS AND CONCENTRATION OF PLASMA Ca AND Mg<sup>a</sup> IN RATS DEPLETED OF MAGNESIUM FOR 14 AND 28 DAYS.

Days of depletion	Parameter	Experiment 1		Experiment 2	
		Control	Mg depleted	Control	Mg depleted
14	Body wt (g)	198 ± 2.9 (23)	192 ± 2.5 <sup>b</sup> (23)	224 ± 4.7 (10)	211 ± 3.1 <sup>b</sup> (10)
14	Plasma Mg (mg%)	2.54 ± 0.08 (16)	1.21 ± 0.07 <sup>b</sup> (16)	2.64 ± 0.14 (6)	1.48 ± 0.08 <sup>b</sup> (6)
14	Plasma Ca (mg%)	10.9 ± 0.4 (3) <sup>c</sup>	11.3 ± 0.5 (3) <sup>c</sup>	10.5 ± 0.2 (7)	11.7 ± 0.3 (7)
28	Body wt (g)	251 ± 3.1 (23)	220 ± 3.0 <sup>b</sup> (23)	—	—
28	Plasma Mg (mg%)	2.43 ± 0.07 (20)	1.04 ± 0.08 <sup>b</sup> (20)	—	—
28	Plasma Ca (mg%)	10.4 ± 0.3 (3) <sup>c</sup>	10.4 ± 0.3 (3) <sup>c</sup>	—	—

<sup>a</sup> Values are means ± SEM; figures in parentheses represent number of rats in each group.

<sup>b</sup>, <sup>c</sup>, <sup>d</sup> Significantly different from control values, *P* < 0.001, *P* < 0.005 and *P* < 0.01 respectively.

<sup>c</sup> Plasma calcium was measured only in three rats which were not used for calcium absorption measurements.

nesium depletion and further increased after 4 weeks (Fig. 1). Uptake of radioactivity by the tibiae generally reflected differences in intestinal Ca transport (Fig. 2). Almost complete transfer of the injected dose had occurred in the duodenal loop 60 minutes after the dose had been injected; less than 60% had been transferred from the ileum in 90 min.

Unidirectional calcium fluxes at different levels of luminal Ca concentration obtained by the *in situ* perfusion method are shown in Fig. 3. Ca influx was consistently less in magnesium depleted rats than in their pair fed controls at all Ca concentrations. How-

TABLE II. COMPOSITION OF THE TIBIA IN RATS MAGNESIUM DEPLETED FOR 28 DAYS AND THEIR PAIR FED CONTROLS.<sup>a</sup>

Parameters	Control	Magnesium deficient
Wet wt. (g)	0.55 ± 0.010	0.60 ± 0.010 <sup>b</sup>
Dry wt. (g)	0.34 ± 0.006	0.34 ± 0.004
Water (%)	38.49 ± 0.65	42.70 ± 0.58 <sup>b</sup>
Mg (meq/tibia)	0.098 ± 0.002	0.047 ± 0.001 <sup>b</sup>
Mg (meq/g dry wt)	0.30 ± 0.004	0.14 ± 0.002 <sup>b</sup>
Ca (meq/tibia)	3.394 ± 0.06	3.60 ± 0.054 <sup>c</sup>
Ca (meq/g dry wt)	10.15 ± 0.13	10.45 ± 0.086 <sup>d</sup>
Mg and Ca (meq/g dry wt)	10.44 ± 0.13	10.58 ± 0.86

<sup>a</sup> Values are means ± SEM of 23 rats in each group.

<sup>b, c, d</sup> Significantly different from control values,  $P < 0.001$ ,  $P < 0.01$  and  $P < 0.05$  levels respectively.

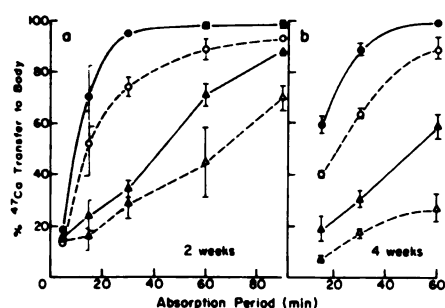


FIG. 1. Effect of Mg deficiency on % <sup>47</sup>Ca transferred to body with time. Each point represents mean ± SEM of Mg deficient rats and pair fed controls. (a) 2 weeks Mg depletion, 2–4 rats/time point; (b) 4 weeks depletion, 4–8 rats/time point. ●—● duodenum, control; ○—○ duodenum, Mg deficient; ▲—▲ ileum, control; △—△ ileum, Mg deficient. Overall rate of absorption was significantly reduced in the duodenum of 14-day depleted rats ( $P < 0.05$ ). The decrease was not significant in the ileum. After 28 days the decrease was significant in both segments,  $P < 0.01$  in the duodenum,  $P < 0.001$  in the ileum.

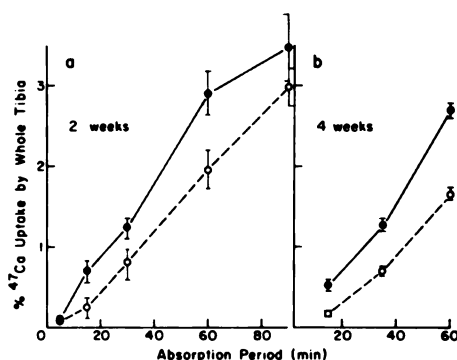


FIG. 2. Effect of Mg deficiency on % <sup>47</sup>Ca uptake by the whole tibia with time. Each point represents mean ± SEM of Mg deficient rats and pair fed controls. (a) 2 weeks Mg depletion, 3–4 rats/time point. (b) 4 weeks Mg depletion, 6–8 rats/time point. ●—● control; ○—○ Mg deficient. Overall <sup>46</sup>Ca uptake was significantly reduced in Mg deficient rats,  $P < 0.01$  after 2 weeks,  $P < 0.001$  after 4 weeks of depletion.

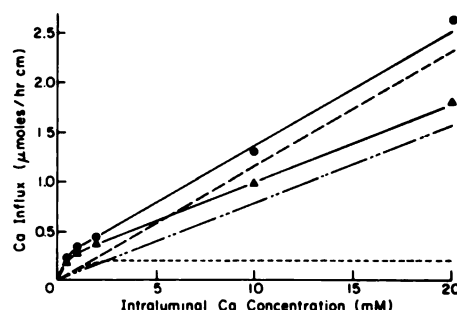


FIG. 3. Effect of 14 days of Mg depletion on Ca transferred from lumen to blood using the *in situ* perfusion technique. Each point represents the mean obtained in 4–9 rats (mean values ± SEM and the number of rats for each point are shown in Table III). ●—● Pair fed control, total <sup>47</sup>Ca transferred ▲—▲ Mg deficient, total <sup>47</sup>Ca transferred --- Pair fed control, linear portion . . . Mg deficient, linear portion ----- Mg deficient and pair fed controls, curvilinear portion with plateau.

ever, the mean values for the data points used to construct Fig. 3, shown in Table III, show statistically significant differences only at luminal Ca concentrations of 0.5, 10 and 20 mM. The relationship between Ca influx and luminal Ca concentration was in agreement with the pattern described by Dumont *et al.* (13), suggesting that Ca absorption in the rat duodenum was comprised of at least two components. The curvilinear portion of the transport-concentration curve, at the lower concentration of calcium, suggests the pres-



TABLE III. MEAN  $\pm$  SEM of Ca TRANSFERRED FROM LUMEN TO BLOOD ( $\mu$ MOLE/Hr  $\text{cm}^{-1}$ ).

Ca conc. (mM)	N	Pair fed control ●—●	Mg deficient ▲—▲
0.5	9	0.22 $\pm$ 0.02	0.11 $\pm$ 0.01*
1.0	8	0.33 $\pm$ 0.03	0.27 $\pm$ 0.03
2.0	6	0.42 $\pm$ 0.06	0.38 $\pm$ 0.05
10.0	5	1.30 $\pm$ 0.22	0.97 $\pm$ 0.16*
20.0	4	2.64 $\pm$ 0.20	1.82 $\pm$ 0.25*

\* Significantly different from control values ( $P < 0.05$ ).

ence of a saturable, carrier mediated mechanism and the linear portion, at higher calcium concentrations, passive diffusion. With this as reference, magnesium depletion appeared to depress passive diffusion of Ca across the duodenal mucosa with also a significant effect at the lowest calcium concentration, 0.5 mM (Table III).

**Discussion.** In the present investigation the depression in Ca transport seen in Mg depleted rats appeared to be entirely due to a decrease in passive diffusion. This finding is in conflict with several previous reports which suggested either an increase or no change in intestinal Ca transport of magnesium-deficient rats (1-5). Of the two previous studies that had shown a decrease in Ca transport (6, 7), one (7) showed an increase in active Ca transport after 10 days of Mg depletion and a significant decrease when Mg depletion was prolonged for 19 days. The transport data in the latter investigation were obtained by an *in vitro* procedure using a modified Ussing apparatus (7). Rats fed adequate magnesium diets showed comparably decreased rates of Ca transport following thyroparathyroidectomy. The authors suggested that both magnesium deficiency and thyroparathyroidectomy depressed Ca transport by alterations in vitamin D metabolism, presumably at the level of regulation of the hydroxylation of 25-OH-D<sub>3</sub> to the 1-hydroxy- or the 24-hydroxy derivatives.

The data reported here do not indicate that Mg depletion of the magnitude or duration applied in this investigation substantially altered vitamin D metabolism. A significant decrease in 1,25-(OH)<sub>2</sub>D<sub>3</sub> should have decreased intestinal Ca absorption by both passive diffusion and saturable transport. While variability may have obscured the significance of differences in Ca transport of defi-

cient and control rats at the luminal Ca concentrations of 1 and 2 mM (Fig. 3), the overall decrease in intestinal Ca transport seen in Mg-depleted animals was small. The Mg deficient diet used in this investigation (50 ppm Mg) was chosen to avoid marked differences in body weights of Mg-depleted and pair fed control rats. Walling *et al.* (7) used a Mg-free diet which probably caused acceleration and enhancement of Mg-depletion and its manifestations, possibly including disturbances in vitamin D metabolism.

Recent findings in this laboratory (14) suggest an explanation for the data reported here which would support the observation of Walling *et al.* (7) that parathyroidectomy and Mg deficiency had similar effects on intestinal Ca transport. Microscopic examination of parathyroid sections removed from Mg deficient rats at intervals from 2 to 21 days of depletion showed progressive manifestations of hypoactivity (14). The same rats consistently exhibited hypercalcemia comparable to that seen in the present investigation in 14 day Mg depleted rats (Table I). Reduction of parathyroid hormone activity is an appropriate response to hypercalcemia. One of the consequences of parathyroid hypoactivity would be depression in intestinal Ca transport.

In conclusion, the decreased rate of intestinal Ca absorption in Mg deficient rats observed in this investigation appears to be due primarily to reduction in the rate of passive Ca diffusion. Among several consequences of magnesium deficiency likely to depress intestinal Ca transport is hypoactivity of the parathyroid glands. This aspect of magnesium deficiency is now under investigation.

**Summary.** Calcium transport across the duodenum and ileum was measured by an *in vivo* ligated loop technique in Mg depleted rats and rats pair fed a magnesium adequate diet. Intestinal Ca transport and tibial <sup>47</sup>Ca uptake were consistently decreased in magnesium depletion. Analysis of Ca fluxes, carried out by *in situ* perfusion, showed a significant decrease in passive diffusion, with less consistent effects on the saturable transport component. Both bone and plasma showed markedly decreased Mg concentration. Tibia Ca levels were slightly but significantly increased and plasma levels were either normal

or slightly, but significantly elevated. The basis for the decrease in Ca transport of Mg depleted rats observed in this investigation is not clear. The data suggest a general alteration in mucosal membrane transport rather than a specific effect on Ca transport *per se*.

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## Anti-Idiotypic Response of BALB/c Mice to a Myeloma Protein of BALB/c Origin (40308)

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It has been demonstrated that a BALB/c myeloma protein with anti-DNP activity (Protein-315) can stimulate anti-idiotypic response in several strains of inbred mice, including the strain from which the plasmacytoma MOPC-315 was originally induced (1, 2). Antibodies produced were found to be specific for the antigen-binding site of Protein-315 (1-3). Tungkanak and Sirisinha (3) also reported that the Fc fragment of Protein-315 was not required for the induction of anti-idiotypic response in BALB/c mice. The anti-idiotypic antibody produced in response to stimulation by the Fab-fragment of Protein-315 was indistinguishable from that produced in response to undigested protein (3). The purpose of the present study was to follow the development of an anti-idiotypic antibody response of BALB/c mice to Protein-315, particularly with regard to the ability of these anti-idiotypic antibodies to compete with the hapten for an antigen-binding site on Protein-315. The results showed that the susceptibility of anti-idiotypic antibody to inhibition by excess hapten (DNP-lysine) depends largely on the immunization procedure used, i.e., the anti-idiotypic antibodies produced following a single booster injection showed a marked increase in the ability to compete with DNP-lysine for the antigen-binding site of Protein-315. Evidence available suggests that this change was associated with an increase in the affinity of the anti-idiotypic antibody produced after a booster injection.

**Materials and methods. Antigens.** Protein-315 and its peptic product (Fv-315) were prepared and purified as described previously

(3). Myeloma sera from BALB/c mice using MOPC-315, MOPC-460, MOPC Adj.PC-22A, J504, and S176 tumors kindly provided by Dr. Herman N. (Massachusetts Institute of Technology, MA).

**Immunization schedule.** BALB/c mice of both sexes used in this study were originally obtained from Jackson Laboratory, Bar Harbor, Maine. Adult mice were immunized at the same time with 200  $\mu$ g of purified Protein-315 at the same two front footpads and four other sites along the back. The procedure of immunization consisted of 3 weekly injections of immunogen in complete Freund's adjuvant, in incomplete Freund's adjuvant, and in potassium phosphate buffered saline (PBS) pH 7.2, respectively. The animals were bled from orbital venous plexus one week after the third injection at weekly intervals thereafter. A booster injection of 200  $\mu$ g of immunogen in PBS was given 1 week after the mice received a full course of primary immunization. The mice had been bled 4 times. These animals were bled again during the 4 succeeding weeks. A similar second booster injection was given to some of these mice and the animals were thereafter bled as described. Individual sera from the same group (5-10 mice/group) were pooled and kept frozen until analyzed.

**Analysis of anti-idiotypic antibody.** An anti-idiotypic antibody to Protein-315 was assayed by radioimmunoassay using <sup>125</sup>I-labeled Protein-315 or Fv-315 as antigen. Pooled sera obtained at weekly intervals were analyzed for their antigen-binding capacity, susceptibility to inhibition by excess hapten, and cross-reactivity with five other myeloma sera of BALB/c origin exactly as described by Tungkanak and Sirisinha (3).

**Results.** The antigen-binding capacity of BALB/c antisera, as determined by their ability to react with <sup>125</sup>I-labeled antigen (Pi

<sup>1</sup> Supported in part by the Rockefeller Foundation.

<sup>2</sup> A portion of this study was submitted by R. T. to the Faculty of Graduate Studies, Mahidol University, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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315 or Fv-315), could be detected as early as 1 week after completion of the primary course of immunization with Protein-315 (Fig. 1). Neither the antigen-binding capacity nor the sensitivity to inhibition by excess hapten altered much during the 13 weeks of observation period. There was also no demonstrable change in specificity as the hapten inhibition values obtained when either Protein-315 or Fv-315 was used as antigen in the assay system were similar (Fig. 1).

The antigen-binding capacity of these antisera was enhanced following a single booster injection with 200  $\mu$ g of Protein-315. As shown in Fig. 2, the quantity of labeled antigen precipitated by 10  $\mu$ l of antiserum increased from less than 40% to more than 60% one week after boosting. It is more interesting however to find that the ability of these post-boosting sera to compete with excess hapten for the antigen-binding site of Protein-315 increased markedly, i.e., within one week the hapten inhibition value decreased from more than 80% to less than 10%, regardless of the type of antigen used in the assay system. Although the susceptibility to inhibition by hapten gradually increased during the next few weeks, the inhibition value did not quite return to the pre-boosting level. Similar but

less obvious changes were observed following a second booster injection.

Selected samples of the pre-boosting (week 4) and post-boosting (weeks 7, 9 and 12) antisera were diluted with pooled normal BALB/c serum and then retested for their susceptibility to inhibition by excess hapten. The results showed that the hapten inhibition values gradually increased as the antisera were being diluted (Table I). The effect of dilution on the hapten inhibition value was independent of the type of the antigen used in the assay system.

Despite a marked change in sensitivity to inhibition by hapten of the antisera obtained

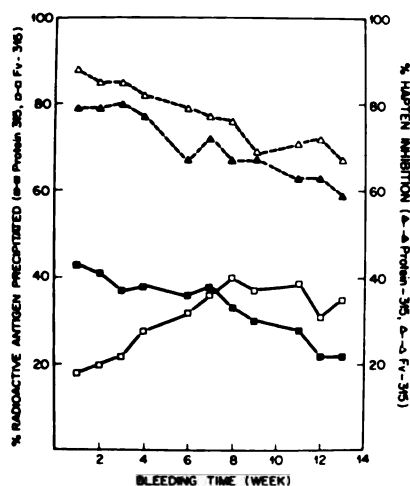


FIG. 1. Antigen-binding capacity and susceptibility to inhibition by excess hapten (750 nanomoles of DNP-lysine) of BALB/c antiserum to Protein-315 from the non-boosting group. Bleeding time represents time after the last injection of the primary course of immunization. Both Protein-315 and Fv 315 were used as antigens in the assay system.

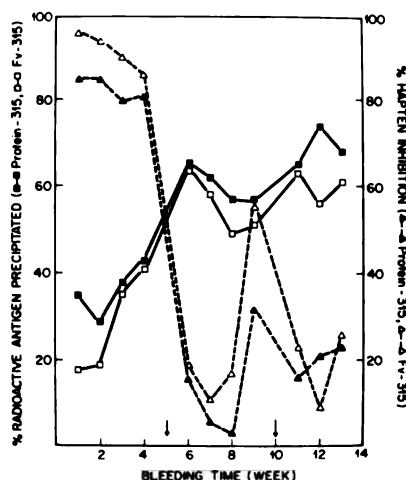


FIG. 2. Antigen-binding capacity and susceptibility to inhibition by excess hapten (750 nanomoles of DNP-lysine) of BALB/c antiserum to Protein-315 from the boosting group. The animals were boosted at weeks 5 and 10 (arrows). See legend to Fig. 1 for other explanations.

TABLE I. EFFECT OF ANTISERUM DILUTION ON HAPTEN INHIBITION<sup>a</sup>

Antiserum	Specimen No. (week)	(% Maximum hapten inhibition (by 750 nmoles DNP-lys)				
		Undilute	1:5 <sup>b</sup>	1:10	1:20	1:40
Preboosting	4	77	87	92	84	90
Postboosting	7	3	53	65	68	68
	9	36	77	81	82	82
	12	12	55	69	73	73

<sup>a</sup> Excess hapten (750 nanomoles) was mixed with 0.5  $\mu$ g of <sup>125</sup>I-labeled Protein-315 before 10  $\mu$ l of antiserum (or its dilution) was added. Thereafter the reaction mixture was treated as described in Materials and methods.

<sup>b</sup> Diluted with pooled normal BALB/c serum.

after boosting, the specificity of these antisera remained unchanged. This was evident from the results of a cross-reactivity study using five other myeloma A sera to inhibit the idiotypic reaction. Like the results obtained with the pre-boosting antisera, the myeloma sera from mice carrying MOPC-460, Adj.PC-22A, and S176 tumors failed to inhibit the anti-idiotypic activity of these postboosting antisera while those from MOPC-292 and J504 mice demonstrated slight inhibition (less than 20%).

**Discussion.** The present observations confirm and extend the original report of Sirisinha and Eisen (1) that under appropriate conditions anti-idiotypic antibody response to a BALB/c myeloma protein with anti-DNP activity can be induced in BALB/c mice. The anti-idiotypic antibody produced is directed largely, if not exclusively, to the antigen-binding site of Protein-315, as evident from the observations that the antisera were highly sensitive to inhibition by excess hapten and they cross-reacted only slightly, if any, with five other myeloma A proteins available for testing.

The interesting feature of the anti-idiotypic response is that a single booster injection not only increased the total antigen-binding capacity of these sera but also markedly decreased their susceptibility to inhibition by excess hapten (Fig. 2). The results obtained following a booster injection are markedly different from those obtained after the primary course of immunization. In the non-boosting group, the antisera obtained at weekly intervals throughout the 13 weeks of observation were equally sensitive to inhibition by hapten and their antigen-binding capacity decreased only slightly during this period (Fig. 1).

The change of the hapten inhibition value obtained after boosting was much larger than can be explained on the basis of a quantitative increase of antibody production by these animals. The reduction of the hapten inhibition value must therefore be attributable to changes in other parameters, e.g., affinity and specificity. Although an increase in affinity of the anti-idiotypic produced after a single booster injection is consistent with the general characteristic of a secondary antibody response (5), the possibility that this could also

be associated with a shift in specificity cannot be completely ruled out. Circumstantial evidence, however, supports the possibility of a marked reduction in the sensitivity to inhibition of these postboosting sera more likely associated with an increase in affinity of antibody. Firstly, the hapten inhibition value of the postboosting sera increased when they were diluted prior to testing (Table I). This interpretation is consistent with the explanation of Sher and Cohen who employed the phosphorylcholine system in their study. Secondly, both the binding capacity and hapten inhibition were similar when either Protein-315 or Fv-315 was employed as antigen in the assay system (2), suggesting that the reaction is primarily restricted to the Fv region. Lastly, the terms of cross-reactivity of the pre-boosting and the post-boosting antisera with myeloma A proteins were indistinguishable from one another (unpublished observations). The possibility that the observed decrease in susceptibility to inhibition by hapten is a laboratory artifact associated with the system employed is unlikely as there was a gradual return of these values toward preboosting level within a few weeks after a booster injection. Likewise, the possibility that there was insufficient labeled antigen in the test system to react with the antibody produced after boosting is also unlikely because under the condition used for assay of antibody, there was excess antigen left in the supernatant fluid. In addition to this evidence, we may add that a booster injection of antigen under identical conditions to other strains of mice (C57BL/6J) failed to cause any reduction of the hapten inhibition value (unpublished observations). It appears from these observations that the insusceptibility to inhibition by excess hapten of the anti-idiotypic produced following a booster injection is more likely associated with an increase in affinity rather than a shift in specificity of these antibodies.

**Summary.** The anti-idiotypic response of BALB/c mice to myeloma protein of BALB/c origin (purified Protein-315 from plasmacytoma MOPC-315) was analyzed for its antigen-binding capacity and susceptibility to inhibition by excess hapten (DNP-lysine). The results showed that the anti-

otypic antibody that is sensitive to inhibition by hapten could be detected for at least 3 months after completion of the primary course of immunization. Following a single booster injection, there was an increase of the antigen-binding capacity and the susceptibility of these post-boosting antisera to inhibition by hapten was markedly reduced (from more than 80% to less than 10% under the assay system employed). However, the hapten inhibition value gradually returned toward the preboosting level within a few weeks. The data obtained suggest that the change in the hapten inhibition value after boosting is associated with increased affinity rather than a shift in specificity.

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## Effects of Indomethacin and Tolmetin on Furosemide-Induced Changes in Renin Release<sup>1</sup> (40309)

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The diuretic furosemide increases renin release by the kidney, an effect independent of volume depletion which accompanies diuresis (1). The stimulus for renin release by furosemide appears to be related to both changes in renal arteriolar resistance (2, 3) and a direct tubular effect subsequent to blockade of sodium and chloride reabsorption prior to or at the macula densa (1, 2).

Furosemide-induced renin release is blocked by the prostaglandin synthetase inhibitor, indomethacin, by an undefined mechanism (4). After indomethacin, the ability of furosemide to increase renal blood flow is blunted, while the natriuretic effect is unaffected (4-6).

Calcium has recently been suggested to play a role in renin secretion (7, 8). Although furosemide is acutely calciuretic, the significance of this effect with respect to renin release has not been evaluated. The purpose of these experiments was to determine if the blockade of furosemide induced renin release by prostaglandin synthetase inhibitors, indomethacin and tolmetin, was correlated with changes in the calciuretic response to furosemide.

**Materials and methods. Surgical.** Male mongrel dogs, 15-25 kg, were used in all experiments. The animals were anesthetized with sodium pentobarbital (30 mg/kg), intravenously, and a cuffed endotracheal tube was inserted. The dogs were artificially ventilated with a Harvard respirator. Catheters were placed in the left femoral artery and in both femoral veins. Normal saline (0.9% NaCl) was infused into one femoral vein to replace fluid losses from surgery and to hydrate the animal until total urine flow was 0.5-2.0

ml/min. The saline infusion was then reduced to equal urine flow. Inulin was infused into the other femoral vein at a rate calculated to maintain plasma inulin concentration between 30-50 mg/dl. Arterial blood pressure was monitored with a Statham P23AC transducer.

**Experimental protocols.** I. Effect of indomethacin on renal responses to intravenous furosemide. Glomerular filtration rate estimated by the clearance of inulin ( $C_{IN}$ ), urinary excretion of Na, K, and Ca and plasma renin concentration (PRC) were measured during two control 10-min clearance periods and during the intravenous infusion of furosemide (2 mg/kg/hr). Following the furosemide clearance periods, each dog received increasing doses of indomethacin (0.01, 0.05, 0.1, 0.5 mg/kg, iv). Furosemide infusion continued during the administration of indomethacin. Twenty minutes after each dose of indomethacin, two clearance periods were obtained. In addition to the dogs treated with indomethacin, three dogs were injected with saline instead of indomethacin in an experimental protocol identical to that described above (4 injections at 40 min intervals). These dogs are referred to as "time" control dogs.

II. Effect of indomethacin or tolmetin pretreatment on renal response to intrarenal furosemide. After two control clearance periods, furosemide was infused (15  $\mu$ g/kg/min) into the renal artery of the experimental kidney, and two clearance periods obtained. The infusion of furosemide was stopped and 30 minutes were allowed for urine flow to return toward control. Two additional control periods were then run and tolmetin (5 mg/kg) or indomethacin (2 mg/kg) was administered intravenously. After 20 min, two more clearances were taken and furosemide again infused intrarenally. Two additional clearance periods were obtained during furosemide infusion. Excretion of Na, K, and Ca, as well

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, and PRC were measured during each 15 min observation period. The dose of furosemide in the tolmetin treated dogs was 5 mg/kg/hr but since the results did not differ from the two dogs given 15 mg/kg/hr the data were pooled.

**Analyses.** Urinary and plasma Na and K were determined by flame photometry and plasma renin activity by atomic absorption spectroscopy. Inulin clearance was determined by the method of Walser *et al.* (9). Plasma renin activity was estimated by incubating plasma with excess homologous renin substrate. The amount of angiotensin I generated was then determined by radioimmunoassay. The data were analyzed utilizing analysis of variance with a randomized block design. The 0.05 level of probability was used as the criterion of significance.

**Results.** Furosemide infused intravenously at 2 mg/kg/hr increased the urinary excretion of sodium and calcium (Table I). Plasma renin concentration (PRC) was also increased. All values remained elevated throughout drug administration (Table I). Glomerular filtration rate was not affected by furosemide. Increasing doses of indomethacin produced dose related decreases in PRC and calcium excretion (Table II). Sodium

excretion and GFR were not changed when indomethacin was given during furosemide infusion (Table II). Potassium excretion (not shown) also increased after furosemide and was not affected by indomethacin.

Furosemide, infused into the renal artery, also increased urinary excretion of sodium, potassium and calcium (Figs. 1 and 2). PRC also increased when furosemide was given. Electrolyte excretions and PRC returned toward control when the infusion of furosemide was stopped. Indomethacin (Fig. 1) and tolmetin (Fig. 2) had little effect on electrolyte excretion although each parameter tended to be lower than the previous control. Similarly, PRC tended to decrease after indomethacin or tolmetin. A second infusion of furosemide increased sodium, potassium, and calcium excretion but PRC was not affected by furosemide after administration of indomethacin (Fig. 1) or tolmetin (Fig. 2).

**Discussion.** Although the role of calcium in renin release is still obscure, there is increasing evidence that movement of this ion within the juxtaglomerular cell may be an important regulatory mechanism. Addition of calcium to kidney slices incubated in calcium free media produces an immediate, large increase in renin release (7). Similarly, the isolated

TABLE I. EFFECT OF TIME ON FUROSEMIDE-INDUCED CHANGES IN RENAL FUNCTION

Parameter	Control	Furosemide <sup>a</sup>	Saline dose			
			1	2	3	4
ml/min) SE	39.9 ± 12.0	29.3 ± 9.7	31.6 ± 10.0	32.7 ± 10.8	31.6 ± 9.4	35.1 ± 9.5
mg AI/ml/hr)	14.9 ± 3.2	38.9 <sup>b</sup> ± 13.1	32.2 <sup>b</sup> ± 13.6	30.0 <sup>b</sup> ± 6.6	30.4 <sup>b</sup> ± 7.1	29.7 <sup>b</sup> ± 3.0
μEq/min) SE	152 ± 32	538 <sup>b</sup> ± 61	649 <sup>b</sup> ± 47	848 <sup>b</sup> ± 92	924 <sup>b</sup> ± 150	717 <sup>b</sup> ± 36
μEq/min) SE	0.61 ± 0.07	11.2 <sup>b</sup> ± 1.9	15.1 <sup>b</sup> ± 3.6	12.6 <sup>b</sup> ± 1.6	10.9 <sup>b</sup> ± 0.9	11.9 <sup>b</sup> ± 3.2

<sup>a</sup> Furosemide was infused at a rate of 2 mg/kg/hr, iv.  
<sup>b</sup> Significantly different than control ( $P < .05$ ).

TABLE II. EFFECT OF INCREASING DOSES OF INDOMETHACIN ON FUROSEMIDE-INDUCED CHANGES IN RENAL FUNCTION

Parameter	Control	Furosemide <sup>a</sup>	Indomethacin dose (mg/kg)			
			0.01	0.05	0.1	0.5
ml/min) SE	43 ± 4	39 ± 4	37 ± 5	36 ± 6	38 ± 7	34 ± 5
mg AI/ml/hr)	16.3 <sup>c</sup> ± 7.1	40.1 <sup>b</sup> ± 9.6	27.6 <sup>b,c</sup> ± 6.8	17.4 <sup>c</sup> ± 4.7	14.5 <sup>c</sup> ± 4.5	13.6 <sup>c</sup> ± 5.5
Eq/min) SE	130 <sup>c</sup> ± 33	844 <sup>b</sup> ± 96	779 <sup>b</sup> ± 56	876 <sup>b</sup> ± 20	854 <sup>b</sup> ± 22.1	656 <sup>b</sup> ± 59
Eq/min) SE	0.80 <sup>c</sup> ± 0.13	17.6 <sup>b</sup> ± 3.2	13.4 <sup>b</sup> ± 1.9	14.1 <sup>b</sup> ± 2.8	10.8 <sup>b,c</sup> ± 2.0	9.8 <sup>b,c</sup> ± 2.2

<sup>a</sup> Furosemide was infused at a rate of 2 mg/kg/hr, iv.  
<sup>b</sup> Significantly different than control ( $P < .05$ ).  
<sup>c</sup> Significantly different than furosemide ( $P < .05$ ).



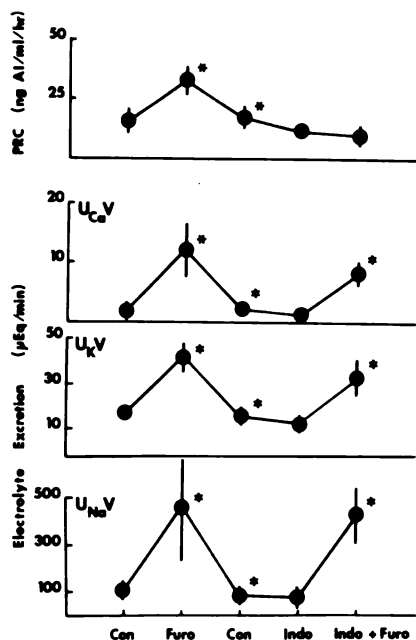


FIG. 1. Effect of indomethacin on renal response to furosemide. Excretion rates of sodium ( $U_{Na}V$ ), potassium ( $U_KV$ ), and calcium ( $U_{Ca}V$ ) and plasma renin concentration were measured. After a control period (CON), furosemide was infused into the renal artery (FURO). After the furosemide infusion was stopped, control values were measured (CON), and indomethacin was given iv (INDO). Forty minutes after indomethacin, furosemide was infused again (INDO + FURO). The mean and 1 SE are given ( $N = 4$ ). \* different from previous clearance period ( $P < .05$ ).

perfused kidney of the cat releases renin in response to calcium only after prior exposure to calcium free perfusate (8). These data indicate that an increase in intracellular free calcium may be involved in renin release.

The present experiments demonstrate that blockade of furosemide-induced renin release by indomethacin or tolmetin does not depend on alterations in net tubular transport of sodium, potassium or calcium. The major stimulus for renin release during furosemide administration appears to be inhibition of sodium (or chloride) flux at the macula densa similar to that observed in the cells of the thick ascending limb of the loop of Henle (1). Since the prostaglandin synthetase inhibitors failed to alter the urinary excretion of sodium in this study or in previous work (4), it is unlikely that the effect of indomethacin or tolmetin on renin release could involve

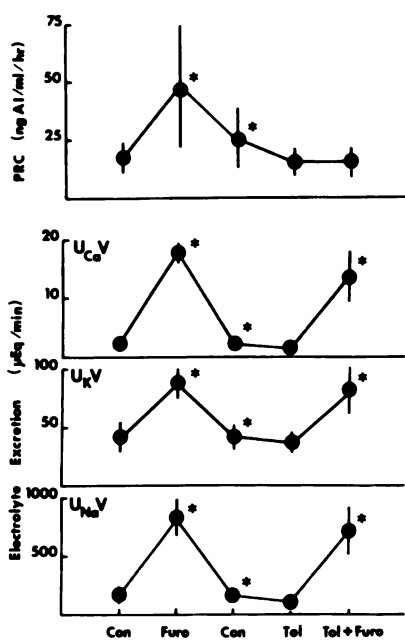


FIG. 2. Effect of tolmetin on renal response to furosemide. Excretion rates of sodium ( $U_{Na}V$ ), potassium ( $U_KV$ ) and calcium ( $U_{Ca}V$ ) and plasma renin concentration (PRC) were measured. After a control period (CON), furosemide was infused into the renal artery (FURO). After the furosemide infusion was stopped, control values were measured (CON), and tolmetin was given iv (TOL). Forty minutes after tolmetin, furosemide was infused again (TOL + FURO). The mean and 1 SE are given ( $N = 4$ ). \* different from previous clearance periods ( $P < .05$ ).

changes in sodium transport at the macula densa.

Similarly, alterations in calcium load to the macula densa do not appear to be important in the action of tolmetin or indomethacin. Although there was a small dose related decrease in calcium excretion after indomethacin, calcium excretion rate was well above control even after the highest dose of indomethacin tested (Table I). In contrast, PRC had decreased dramatically. In addition, pretreatment with neither indomethacin nor tolmetin altered the increase in calcium excretion to intrarenal furosemide, while both drugs blocked any increase in PRC (Figs. 1 and 2). Thus, blockade of furosemide-induced renin release by prostaglandin synthetase inhibitors does not require an alteration in the calciuretic effect of furosemide. Lester and Rubin also found extracellular calcium

was not a determinant in the release of renin following furosemide (8). Since prostaglandin synthetase inhibitors, such as indomethacin or tolmetin, do not appear to affect sodium or calcium load at the macula densa, their site of action is probably subsequent to the signal perceived by the macula densa. Whether their action involves alterations in the state of intracellular calcium remains to be investigated.

**Summary.** Prostaglandin synthetase inhibitors, indomethacin and tolmetin, blocked furosemide-induced increase in renin secretion whether the furosemide was given intravenously or into the renal artery. Tolmetin and indomethacin did not affect the natriuretic, kaliuretic or calciuretic response to furosemide. Therefore, blockade of furosemide-induced renin release does not appear to require an alteration in sodium or calcium load at the macula densa. Thus, the site of action of prostaglandin synthetase inhibitors on renin release is probably subsequent to the signal perceived by the macula densa.

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# Absence of Cytotoxic Effect of Selected Pathogens on HLA B27 Positive Fibroblasts (40310)

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There is strong suggestive evidence that at least one of the seronegative spondyloarthropathies, Reiter's disease (RD), follows genital exposure to certain infectious agents including *Chlamydia trachomatis* and *Ureaplasma urealyticum* (1). In addition, postdysenteric RD and similar forms of acute reactive arthritis are known to follow enteric infections with Enterobacteriaceae such as *Salmonella* and *Yersinia* (2). Eventually, some patients with these acute post-infective arthropathies may develop chronic sequelae identical to those seen in ankylosing spondylitis (AS) (1).

It is now firmly established that these same seronegative spondyloarthropathies are strongly associated with the B-locus histocompatibility antigen HLA B27 (3). Thus it appears that exposure to certain specific microbial agents in a genetically susceptible host may be a prerequisite for the development of this spectrum of acute-to-chronic rheumatic disease.

Several possibilities emerge from this concept. Firstly, the B27 antigen present on cell surfaces might facilitate microbial attachment and invasion. Secondly, the cell surface antigen B27 might share antigenic similarities with the microbial agents initiating these diseases. Under this circumstance the body's host defense mechanism may not recognize the agents as foreign and antigenic. Thirdly, the chromosomal locus which codes for HLA B27 is located within the major histocompatibility complex (MHC) region of the sixth autosomal chromosome. There is strong evidence in other species and suggestive evidence in man that immune response genes are also located within the same genetic complex, and that such genes may be linked to the HLA loci (4). Conceivably, immune responsiveness controlled by HLA-linked genes may be responsible for the development and/or propagation of connective tissue inflammation typified as RD and AS.

A first step in elucidating the potential role

of MHC gene products in RD and to explore their influence on cell surface reactivity to implicated pathogens. Using a diacytotoxicity assay previously standardized by cell counts and correlated to dye exclusion, the cytotoxicity of implicated pathogens to human cells was investigated. The experiments reported here indicate that HLA B27 has no such direct role in initiating the lesion of the seronegative spondyloarthropathies.

**Material and methods. Target cells.** Skin fibroblasts were cultured from 40 depth punch biopsies of normal and arthritic volunteers with RD or AS who had been typed for the absence or presence of HLA B27. It has been demonstrated that fibroblasts of B27-positive individuals retain the surface markers for at least 12 weeks (5, 6). Explants (1 mm<sup>3</sup>), devoid of epidermis and subcutaneous fat, were secured in culture flasks (Falcon, Oxnard, CA) under the surface tension of the culture medium. Cells were established in Eagle's BME (Gibco, Grand Island, NY) supplemented with penicillin 100 units/ml, streptomycin 100 µg/ml, L-glutamine and 15% unheated fetal calf serum (Gibco, Grand Island, NY), and kept at 37°C. Medium was changed weekly for the first 2 weeks, twice weekly thereafter. At first change, all further culture was in the absence of antibiotics. At 4-5 weeks, confluent fibroblast monolayers were trypsinized (0.25% in Hanks (HBSS; Gibco, Grand Island, NY), pH 8.2, 5' at 37°C) and subcultured in 1:2 splits. After four subcultures, cells were assayed for bacterial and mycological contamination. Fibroblasts for cytotoxicity targets were harvested at late logarithmic phase and used only within 5th-20th passages.

**Pathogens.** The following pathogens were obtained from sources indicated, were cultured by standard methods. At least three

tures preceded the final effector organisms used to assay cytotoxicity. Identity of later subcultures was reconfirmed by source labs.

<i>Yersinia enterocolitica</i>	type 8
<i>Salmonella minnesota</i>	595
Cytomegalovirus	AD-169
Herpes simplex virus	type 2
<i>Ureaplasma urealyticum</i>	T-960
<i>Mycoplasma hominis</i>	1001
<i>Chlamydia trachomatis</i>	UW-3 (type D)
<i>Chlamydia trachomatis</i>	UW-5 (type E)

are expressed as fractional  $^{51}\text{Cr}$  release. In Fig. 2, the  $^{51}\text{Cr}$  release effected by  $\text{dH}_2\text{O}$  is illustrated as specific cytotoxicity by relating

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**Cytotoxicity assay.** Fibroblast cultures were trypsinized into fresh culture medium and adjusted to  $1.0 \times 10^5$  cells per ml.  $1 \mu\text{Ci/ml}$   $\text{Na}_2^{51}\text{CrO}_4$  (ICN, Irvine, CA) was added and the suspension distributed in 1 ml aliquots to sterile flat-bottomed glass tubes (Cal Gass 15-105) which could subsequently be inserted in a well-type Nuclear Chicago  $\gamma$ -counter. Gas phase of each tube was equilibrated with 5%  $\text{CO}_2$  in air, and the tubes were capped and incubated at  $37^\circ$ . At 16 hr the adherent fibroblast monolayers were washed with 1 ml/tube HBSS containing 10% FCS. After 1 more hr the wash was repeated, and the monolayers were covered with 1 ml of fresh culture medium. 0.10 ml of selected log-phase pathogen, adjusted to proper multiplicity of infection (MOI), was added to each tube. Positive controls received 6N HCl; negative controls received medium only. At intervals after the addition of pathogens, supernatants were transferred to separate  $\gamma$ -counter tubes, the remaining monolayers were gently rinsed with 5% FCS in HBSS, and the rinses were pooled with supernatants. Tubes with media and tubes with cells were counted for  $\gamma$ -emission. Fractional  $^{51}\text{Cr}$  release (FR) for each cell-medium pair was expressed as

$$\frac{\text{CMP}(\text{medium})}{\text{CMP}(\text{cells}) + \text{CMP}(\text{medium})}$$

and specific cytotoxicity of each pathogen as

$$\frac{\text{FR}(\text{pathogen}) - \text{FR}(\text{spontaneous})}{\text{FR}(\text{maximum})}$$

Figures 1 and 2 illustrate the course of a prototype assay, used to determine suitable levels of target cell label. In Fig. 1, spontaneous, intermediate, and maximum  $^{51}\text{Cr}$  release are achieved in culture medium, 70% distilled water ( $\text{dH}_2\text{O}$ ), and 0.1 M sodium dodecyl sulfate (SDS) respectively. Results

it to a baseline represented by spontaneous release.

**Results.** Table I shows the mean fractional  $^{51}\text{Cr}$  release of three B27-negative and three B27-positive fibroblast strains exposed to each pathogen. In Table II the specific cytotoxicity of each pathogen on these target fibroblast strains has been calculated. Over the range of dilutions used ( $10^4$ ,  $10^0$ , and  $10^{-4}$  MOI), paired  $t$  tests revealed no significant difference in the cytotoxic effect of any one pathogen on B27-positive compared to B27-negative fibroblasts with the apparent exception of *Ureaplasma urealyticum*. However, the differential killing for this organism is almost certainly not significant since it only repre-

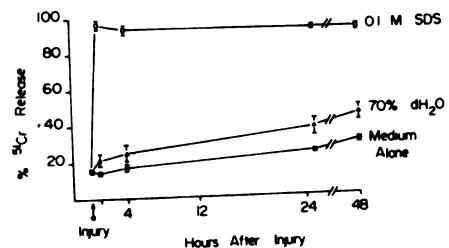


FIG. 1. Fractional  $^{51}\text{Cr}$  release. Cytotoxic effect of 70%  $\text{dH}_2\text{O}$  on normal human skin fibroblasts. Data points represent mean  $\pm$  SEM of three determinations.

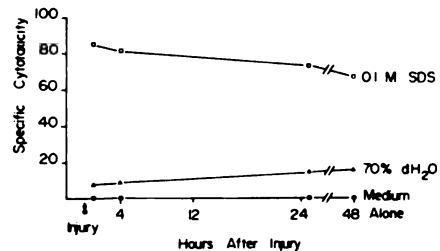


FIG. 2. Specific cytotoxicity. Results of Fig. 1 adjusted to spontaneous release baseline. Data points represent  $\text{FR}(X) - \text{FR}(\text{Spontaneous}) / \text{FR}(\text{Maximum})$ .

TABLE I. MEAN FRACTIONAL  $^{51}\text{Cr}$  RELEASE OF B27- AND B27 FIBROBLASTS IN PRESENCE OF PATHO

Viable organism	Cytotoxicity read at hours	Mean fractional $^{51}\text{Cr}$ release $\pm$ SEM*				
		$10^4$ MOI		$10^0$ MOI		$10^{-4}$ MOI
		B27-	B27	B27-	B27	B27-
<i>Y. enterocolitica</i> type 8	24	.626 $\pm$ .057	.683 $\pm$ .074	.444 $\pm$ .062	.578 $\pm$ .092	.411 $\pm$ .072
<i>S. minnesota</i> 595	48	.929 $\pm$ .017	.876 $\pm$ .004	.913 $\pm$ .013	.891 $\pm$ .016	.912 $\pm$ .022
CMV AD-169	24	.177 $\pm$ .030	.180 $\pm$ .059	.166 $\pm$ 0.29	.119 $\pm$ .018	.194 $\pm$ .026
HSV-2 #1000	24	.162 $\pm$ .033	.154 $\pm$ .036	.194 $\pm$ .045	.110 $\pm$ .005	.234 $\pm$ .060
<i>U. urealyticum</i> T-960	18	.213 $\pm$ .063	.164 $\pm$ .008	.219 $\pm$ .080	.172 $\pm$ .009	.211 $\pm$ .027
<i>M. hominis</i> #1001	18	.245 $\pm$ .081	.170 $\pm$ .004	.183 $\pm$ .024	.187 $\pm$ .025	.169 $\pm$ .019
<i>C. trachomatis</i> UW-3 (D)	24	.245 $\pm$ .024	.217 $\pm$ .013	.246 $\pm$ .024	.244 $\pm$ .011	.255 $\pm$ .029
<i>C. trachomatis</i> UW-5 (E)	24	.242 $\pm$ .030	.246 $\pm$ .027	.246 $\pm$ .039	.214 $\pm$ .008	.254 $\pm$ .036

\* Three experiments were performed.

\* One experiment performed, due to technical difficulties.

TABLE II. SPECIFIC CYTOTOXICITY OF PATHOGENS ON B27- AND B27 FIBROBLASTS. SIGNIFICANC

Viable organism	Cyto- toxicity read at hours	Specific cytotoxicity						Paired T
		10 <sup>4</sup> MOI		10 <sup>0</sup> MOI		10 <sup>-4</sup> MOI		
		B27-	B27	B27-	B27	B27-	B27	
Y. enterocolitica type 8	24	.289	.108	.084	-.011	.046	-.023	1.538
S. minnesota 595	48	.326	.312	.309	.328	.308	.322	-0.583
CMV AD-169	24	.012	.050	-.001	-.024	.032	.028	-0.222
HSV-2 #1000	24	-.006	-.169	.032	-.035	.079	.017	2.939
U. urealyticum T-690	18	.037	.010	.043	.018	.035	.001	10.74
M. hominis #1001	18	.069	.016	.007	.033	-.007	.017	0.038
C. trachomatis UW-3 (D)	24	-.020	-.008	-.019	.019	-.010	-.010	1.545
C. trachomatis UW-5 (E)	24	-.023	.021	-.019	-.011	-.011	.033	-2.667

sents a difference between 1 and 2% nonspecific versus 3 and 4% specific cytotoxicity. The  $t$  value is large because the two ranges were very small and did not overlap. The different pathogens damaged targets to different extents, but none truly differentiated between B27-negative and B27-positive fibroblasts.

**Discussion.** Over 90% of patients with RD or AS bear B27 on their cell surface. Conceivably, the presence of this membrane polypeptide might render the cells more vulnerable to direct attack by certain pathogens, notably those temporally incriminated with patients with RD and other post-infective arthropathies. Such susceptibility would permit rapid attachment, invasion, and cell destruction before host immune recognition and defense were fully mobilized. However, our study shows that B27-positive skin fibroblasts do not differ from B27-negative skin fibroblasts with respect to their susceptibility to damage by selected pathogens.

Other relevant organisms assayed in other *in vitro* systems may possibly demonstrate an enhanced and differential cytotoxicity toward

B27-positive target cells, but this would appear to be unlikely.

**Summary.** A sensitive index of *in vitro* damage has been used to investigate the possibility that HLA B27-positive fibroblasts are peculiarly susceptible to those infectious agents incriminated in the seronegative spondyloarthropathies. No evidence for differential susceptibility related to the presence or absence of the B27 antigen could be demonstrated.

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## Effect of Ethanol on Parathyroid Hormone and Calcitonin Secretion in Man (40311)

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Peng *et al.* (1, 2) showed that ethanol can induce hypocalcemia in dogs and in intact and parathyroidectomized rats, which could not be prevented by exogenous parathyroid hormone (PTH). Ramp *et al.* (3) observed ethanol-induced hypocalcemia in chickens. Subsequent studies in the rat from our laboratory (4) showed that ethanol caused a dose related hypocalcemia and an increase in PTH secretion which, however, was not sufficient to correct the hypocalcemia. The present study (a) evaluated the effect of ethanol on PTH and calcitonin (CT) secretion *in vivo* in normal man, and (b) evaluated the mode of ethanol effect on PTH secretion by studying its effect on bovine parathyroid tissue *in vitro*.

**Materials and methods. Human studies.** Normal male subjects aged 25-50 years, on normal diets and with no evidence of renal, calcium (Ca) metabolic or other endocrine abnormalities underwent the alcohol ingestion test. Informed consent was obtained from each subject to undergo this procedure, which had been approved by the Human Investigation Committee of this institution. The subjects were fasted for 12-15 hr before the procedure and were recumbent during the procedure. A scalp vein needle was placed in an antecubital vein and attached to an infusion set via a 3-way stopcock for the slow administration of normal saline (0.5 ml/min) and withdrawal of serial blood specimens. After a 30 min rest period, blood specimens were obtained for plasma immunoreactive (i) CT and for serum iPTH and Ca at -10 and -5 min for baseline values. The subject then drank ethanol (0.8 g/kg) in the form of 86 proof bourbon whiskey (one fourth of total dose at 0, 20, 40 and 60 min). Additional blood specimens were obtained at ½, 1, 1½,

2, 3, 4 and 4½ hr from the time ethanol ingestion was begun. A control group of five normal male subjects underwent a procedure which was similar except that ingestion of a volume of tap water approximating the volume of ethanol was substituted for the ingestion of ethanol.

A portion of each blood specimen was placed in a chilled heparinized tube, centrifuged in a refrigerated centrifuge and the plasma separated and frozen immediately for subsequent analysis of iCT. The other portion of the blood specimen was placed in a plain tube, allowed to clot for 1 hr, centrifuged, and the serum separated and frozen for subsequent iPTH and Ca determination.

Serum iPTH was determined by a method developed in this laboratory (5) using a guinea pig antbovine PTH antiserum, purified bovine PTH (Wilson Laboratories, lot 147865) for tracer, and dilutions of a pool of human parathyroid tissue culture medium for standards. This antiserum detects both the intact molecule and the amino terminal fragment of bovine iPTH, its molar affinity for bovine PTH 1-34 being approximately one half that for the intact PTH 1-84 molecule in the utilized portion of the standard curve. This antiserum has a high affinity for human and monkey iPTH, and detects dilutions of human serum and purified bovine PTH with superimposable displacement curves over a 60-fold dilution span. The normal mean value for human serum iPTH is 6.2 µl eq standard human parathyroid tissue culture medium/ml (µl eq/ml) with a normal range (mean ± 2 SD) of 3.8-8.6 µl eq/ml.

Plasma iCT was determined by a method developed in this laboratory (6, 7) using a goat antihuman synthetic CT antiserum and human synthetic CT (N.V. Organon, batch #SC 30) for standard and tracer. The normal mean value for human adult male plasma

<sup>1</sup> Supported by the Medical Research Service of The Veterans Administration.

iCT is 218 pg/ml with a normal range (mean  $\pm$  2 SD) of 55–380 pg/ml.

Ethanol was added to serum and plasma specimens to a concentration of 1.6%, allowed to incubate at 4° for 2 hr and then assayed for iPTH and iCT respectively to determine whether ethanol may cause any degradation of these hormones or modification of the displacement of tracer, which would modify the assay-detected concentrations of iPTH or iCT.

Serum Ca concentration was determined by a modification of the method of Hill (8). The normal mean value is 9.2 mg/dl with a normal range of 8.2–10.2 mg/dl.

**In vitro studies.** Fresh bovine parathyroid tissue slices were incubated for 4 hr in Eagle Minimal Essential Medium with 10% calf serum by the technique previously described from this laboratory (9). The medium was completely aspirated and replaced by fresh medium hourly. During the first 2 hr the medium in all flasks contained 1.25 mM Ca (considered to approximate the ionized Ca concentration of normal plasma). The first hr of incubation was considered an equilibration period and this medium was discarded. The iPTH in the medium removed at the end of the next hr was considered to represent the control or zero-time baseline secretion of the tissue in that flask. The composition of the medium was then modified to contain either a high (3.0 mM) or a low (0.75 mM) Ca concentration or to contain either 0.05% or 0.3% ethanol, and incubation was continued for 2 additional hr. The iPTH concentration of each hourly medium sample was determined by radioimmunoassay as previously described (9), using purified bovine PTH for standard and tracer. The concentration of iPTH in pg/mg wet wt of parathyroid tissue in the zero-time baseline medium sample of each flask was designated as 100%. The iPTH concentration in the medium harvested at the end of each hr for the next 2 hr was then expressed as a percent of this zero-time baseline value for that flask (9). At least three control flasks containing 1.25 mM Ca during the entire incubation period were included with each group of incubation flasks to evaluate uniformity of secretion with time. The percent of zero-time baseline values obtained on hours 1 and 2 with the control flasks were

then adjusted to 100%, and the data of the other flasks corrected to this as baseline. Also, aliquots of media in without tissue were assayed to determine whether the ethanol had any nonspecific effects on the immunoassay results.

In all studies the mean and SE of the time period were calculated from the actual percent values for the time period of each subject (*in vivo* studies) or each *in vitro* studies). Statistical tests of significance were carried out with Student's *t* test.

**Results. Human studies.** The mean values (mean  $\pm$  SE) for the six human subjects were: iPTH–6.6  $\pm$  0.30  $\mu$ l eq/ml, iCT–269  $\pm$  24.5 pg/ml, Ca–9.0  $\pm$  0.11 mg/dl. The direct addition of ethanol to serum or plasma caused no change in iPTH or iCT concentrations from those observed in the serum or plasma without addition of ethanol. As indicated in Fig. 1, infusion of normal saline and ingestion of water by normal subjects caused no change in iPTH or Ca. However, as shown in Fig. 2, infusion of ethanol caused a significant (*P* < 0.05) increase in iPTH to 107.2  $\pm$  2.11% of baseline by 30 min, at a time when only half the ethanol had been ingested. The iPTH secretion continued to increase, reaching a value of 138.9  $\pm$  4.44% of baseline (*P* < 0.05) at 2 hr, with gradual decrease thereafter to 106.0  $\pm$  8.10% of baseline at 4½ hr.

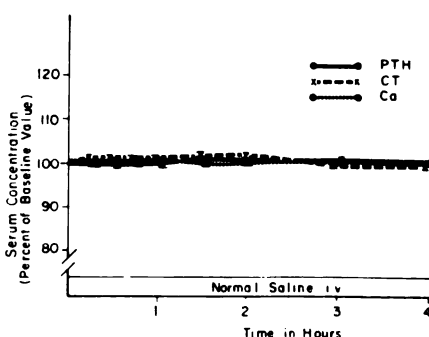


FIG. 1. Effect of iv infusion of normal saline on serial serum iPTH, iCT, and serum Ca concentrations during a 4½-hr period in a normal man. Values (mean  $\pm$  SE) at each time are expressed as percent of the baseline pre values (designated as 100%). *N* = 5. Baseline iPTH – 6.4  $\pm$  0.28  $\mu$ l eq/ml, iCT – 240  $\pm$  22 pg/ml, Ca – 9.3  $\pm$  0.12 mg/dl.

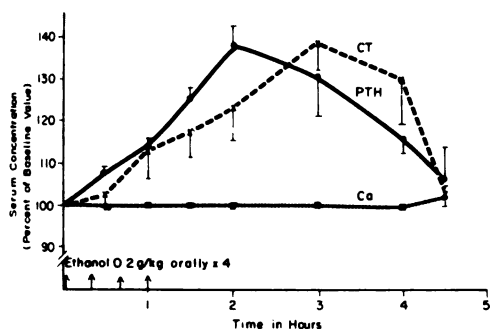


FIG. 2. Effect of ethanol ingestion on serial serum iPTH, plasma iCT and serum Ca concentrations during the following 4½ hr in normal man. Values (mean  $\pm$  SE) at each time period are expressed as per cent of the baseline pre-ingestion values (designated as 100%).  $N = 6$ . Baseline values: iPTH —  $6.6 \pm 0.30$   $\mu$ l eq/ml, iCT —  $269 \pm 24.5$  pg/ml, Ca —  $9.4 \pm 0.11$  mg/dl.

iCT concentration increased more slowly, showing a significant ( $P < 0.05$ ) rise to  $115.0 \pm 6.07\%$  of baseline at 1½ hr, reaching a peak value of  $137.8 \pm 7.13\%$  of baseline ( $P < 0.001$ ) at 3 hr and then decreasing to  $101.9 \pm 1.90\%$  of baseline at 4½ hr. Serum Ca did not significantly change at any time tested.

**In vitro studies.** Aliquots of medium (with or without added ethanol) which had been incubated without parathyroid tissue revealed no modification of the trace B/F ratio, indicating that neither the medium nor ethanol had any nonspecific effects on the immunoassay results. Changes in *in vitro* secretion of iPTH, related to medium changes in Ca ion concentration or to addition of ethanol, are portrayed in Fig. 3. Hourly iPTH secretion revealed only minimal variation when medium containing 1.25 mM Ca was used during the entire incubation period: (iPTH =  $325 \pm 13.8$ ,  $311 \pm 22.4$  and  $319 \pm 14.0$  pg/mg wet wt of parathyroid tissue/hr at 0, 1 and 2 hr respectively). Each value was designated at 100% for that hr. Medium containing low (0.75 mM) Ca caused a significant ( $P < 0.001$ ) increase in iPTH release to  $142.5 \pm 9.41\%$  and  $240.2 \pm 8.10\%$  of baseline at the first and second hr of incubation respectively. Medium containing high (3.0 mM) Ca caused a significant ( $P < 0.001$ ) decrease to  $57.3 \pm 4.63\%$  and  $42.7 \pm 4.23\%$  of baseline at the first and second hr of incubation respectively. Addition of two concentrations of ethanol to 1.25 mM Ca medium caused increases in

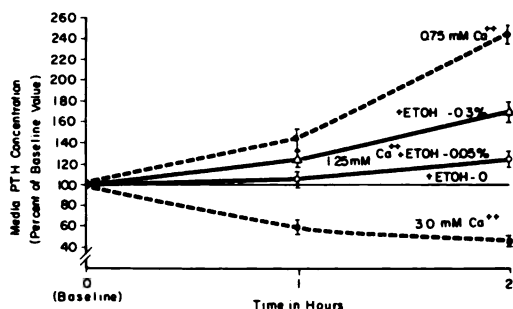


FIG. 3. Effect of Ca concentration and of concentrations of ethanol on hourly iPTH secretion *in vitro* by normal bovine parathyroid tissue. Medium prior to zero time contained 1.25 mM Ca and no ethanol in all flasks. When medium containing 1.25 mM Ca was continued during the entire incubation period (control flasks), the iPTH values (mean  $\pm$  SE) were:  $325 \pm 13.8$ ,  $311 \pm 22.4$  and  $319 \pm 14.0$  pg/mg wet wt parathyroid tissue at 0, 1 and 2 hr respectively. Each of these values was designated as 100% baseline for that hr. Each value is expressed as per cent of the zero time iPTH secretion for that flask, (corrected for the minimal variation of secretion in control flasks for that hr). Each point is the mean  $\pm$  SE for six flasks.

iPTH secretion. At a concentration of 0.05% ethanol, the increase to  $105.1 \pm 6.10\%$  of baseline at 1 hr was not significantly different from baseline, but the iPTH increase to  $122.1 \pm 6.74\%$  at 2 hr was significantly ( $P < 0.02$ ) increased. The 0.3% concentration of ethanol caused increases in iPTH secretion to  $124.1 \pm 6.35\%$  at 1 hr and to  $166.3 \pm 11.26\%$  of baseline at 2 hr, both being significantly ( $P < 0.02$  and  $P < 0.001$  respectively) increased from baseline secretion.

**Discussion.** Our initial studies of the effect of ethanol on Ca metabolism in the rat (4) suggested that decrease in serum Ca was the primary event and the observed increase in serum iPTH was in response to the hypocalcemic effect of ethanol. However, this compensatory increase in iPTH did not prevent or fully correct the hypocalcemia. We proposed (through without supporting data) that ethanol may induce a decrease in bone resorption, leading to hypocalcemia and a relative skeletal resistance to the resorptive action of PTH. However, the studies of Peng *et al.* (2) suggest that decreased bone resorption does not occur and that there may be a shifting of Ca from extracellular fluid into tissues to explain the hypocalcemia. This ex-



planation is strengthened by the observations of Ramp *et al.* (3) that adding ethanol to the organ culture medium enhanced mineral accretion by embryonic chick bone.

The present study indicates that, in normal man, ethanol induces an increase in both serum iPTH and iCT without detectable change in plasma Ca. This observation could be explained by ethanol-induced decrease in bone resorption or increase in bone accretion, but without skeletal resistance to PTH. In this situation very minimal hypocalcemia would induce increased PTH secretion, with rapid bone resorption and restoration of serum Ca to normal, so that hypocalcemia was never detectable. However the *in vitro* observations indicate that a primary change in serum Ca is not the total explanation of the changes in serum iPTH. In this situation ethanol had a direct stimulatory effect on the parathyroid cell which was dose-related. It is therefore possible that ethanol has both an indirect (via induced hypocalcemia) and a direct effect on PTH secretion. The effect of ethanol on the C cell of the thyroid was not studied *in vitro*, but is inferred to also be direct, leading to increase in CT secretion. The simultaneous increase in PTH and CT secretion may at least partially explain the lack of changes in serum Ca in the present study.

Other investigators have reported that ethanol can stimulate CT secretion in patients with medullary carcinoma of the thyroid, and have proposed ethanol ingestion as a CT secretagogue (along with calcium infusion and pentagastrin injection) as a diagnostic test for this tumor (10-14). Initially, it could not be demonstrated that ethanol affected CT secretion in normal subjects (13), but a subsequent study, using a more sensitive assay method, demonstrated that some normal subjects do show a CT response to ethanol (14). In the present study using a larger dose, ethanol elicited a CT response in all six normal subjects tested. We are not aware of a previous report of the effect of ethanol on PTH secretion.

The lower media concentration (0.05%) of ethanol in the *in vitro* study is comparable to the average blood ethanol concentration achieved in social drinking situations, and the 0.3% media concentration of ethanol is comparable to the blood ethanol level achieved

by severely intoxicated subjects (15). Doses of ethanol (0.8 g/kg) ingested by human subjects in the present study rendered them only moderately intoxicated. The ethanol, in amounts often ingested by drinkers, increases both PTH and CT secretion, and therefore may modify Ca homeostasis.

**Summary.** Ingestion of 0.8 g/kg ethanol 1 hr by normal man caused significant increases in both serum PTH and plasma concentrations, with peak values of 150% of baseline at 2 hr for PTH and of 138% for CT. Serum Ca did not change during the period of observation. Incubation of parathyroid slices in 1.25 mM Ca Eagle medium with 0.05% or 0.3% ethanol caused significant increases in PTH secretion to 122% and 166% of baseline respectively. Therefore *in vitro*, ethanol can be demonstrated to directly stimulate PTH secretion, (2) *in vivo* ethanol ingestion induces an increase in PTH secretion without detectable hypocalcemia, suggesting (a) prompt PTH secretion and action to compensate for a hypocalcemic effect of ethanol so that actual hypocalcemia is not detected and/or (b) direct parathyroid stimulation. Though the exact mechanisms are unclear, the data indicate that ethanol, in amounts often ingested by social drinkers, increases both PTH and CT secretion, and therefore may modify Ca homeostasis.

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## Pancreatic Secretory Isoenzyme of Alkaline Phosphatase (40312)

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Alkaline phosphatase exists in a wide variety of tissues in different molecular forms. Characterization of these isoenzymes is possible on the basis of their resistance to various physical and chemical manipulations. As early as 1944, Nothmann (1) reported that ligation of the pancreatic duct in dogs produced an increase in serum alkaline phosphatase, but there have been few attempts to measure this enzyme in pancreatic juice. Warnes and Bulmer (2) demonstrated the presence of alkaline phosphatase in the duct system, islet cells, and acini of the human pancreas. Warnes *et al.* (3) extracted alkaline phosphatase from normal human pancreas and pancreatic tumors and showed that these enzymes have distinctive isoenzyme characteristics when compared with the enzymes of the small intestine and of normal serum.

The present study was designed to examine the isoenzyme characteristics of alkaline phosphatase in canine pancreatic secretory fluid. The availability of pure human pancreatic juice from a patient with a traumatic fistula allowed us to conduct similar observations in this fluid.

**Methods.** Six adult mongrel dogs, weighing 14-18 kg, were previously prepared with gastric and pancreatic fistulas fitted with Thomas cannulas in the stomach and duodenum (4). Animals were not studied until 3-4 weeks after this operation and were deprived of food but not water for approximately 18 hr prior to each study. A continuous iv infusion of 0.15 M sodium chloride was given at a rate of 50 ml/hr. Observations were carried out in conscious animals during continuous intravenous infusion of secretin, 0.5 U/kg per hr. The secretin used in these studies was from a single batch purchased from the Gastrointestinal Hormone Research Unit, Karolinska Institute Chemistry Department,

Stockholm, Sweden. The gastric cannula was kept open during all observations to prevent the entry of acid into the duodenum. The duodenal cannula was opened and a glass cannula was inserted into the pancreatic duct under direct vision. Pancreatic secretion was collected continuously as 10-min specimens.

Pancreatic juice also was collected from a patient who had an established posttraumatic pancreatic fistula that was draining clear, alkaline juice, 400-600 ml/day, with a bicarbonate concentration of 68 meq/liter and an amylase concentration of 120,000 Somogyi U/100 ml. Fluid was collected by direct cannulation of the fistula with a sterile catheter after appropriate skin cleansing to minimize the likelihood of bacterial contamination.

Alkaline phosphatase, expressed in international units, was determined by the method of Roy (5) with thymolphthalein monophosphate as the substrate.

Isoenzyme characterization, based on different susceptibilities of alkaline phosphatase isoenzymes to inhibition by urea and L-phenylalanine (6-8) and heat inactivation (9), was performed in all specimens. The method of Kind and King (10) was used for alkaline phosphatase measurements in these isoenzyme studies.

Isoenzymes present in the human pancreatic fistula fluid were examined by acrylamide gel electrophoresis and compared to the electrophoretic behavior of alkaline phosphatase of known human origin from liver, bone, and intestine. Liver alkaline phosphatase was obtained from the serum of patients with known liver disease and intestinal alkaline phosphatase was purchased from Dade Corporation. Bone alkaline phosphatase was obtained from shavings of bone extracted with butanol to remove insoluble material and break the protein-lipid bond. The alkaline phosphatase obtained from the pancreatic fistula fluid was concentrated ten

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efore electrophoresis. All samples to be  
phoresed were dialyzed for twelve  
against two changes of electrophoresis  
Alkaline phosphatase isoenzymes  
eparated by Raymond's method of cons  
polyacrylamide gel electrophoresis in  
ical cell (11).

ults. At the low dose of secretin infusion  
d in the canine studies, pancreatic se-  
y volumes varied from 5 to 10 ml/10  
The mean ( $\pm$  SEM) alkaline phosphatase  
oncentration in specimens from all six  
ds (87 collections) was  $15.4 \pm 1.1$   
nl. Alkaline phosphatase concentration  
reatic fistula fluid collected from the  
it was 17.8 mU/ml.

ure 1 shows the percentage of alkaline  
hatase remaining in pancreatic juice  
each of the six dogs after incubation of  
ens with urea or phenylalanine or after  
activation. There was relative uniform-  
ong the animals in that the isoenzyme  
ted relative resistance to phenylalanine  
tion, intermediate inhibition by urea,  
arked thermal lability.

enzyme characteristics of alkaline phos-  
se in canine and in human pancreatic  
ry fluid are compared in Fig. 2. The  
alkaline phosphatase activity remain-

ing after phenylalanine inhibition was 67% in  
canine pancreatic juice compared to 85% in  
human juice. The mean values after urea

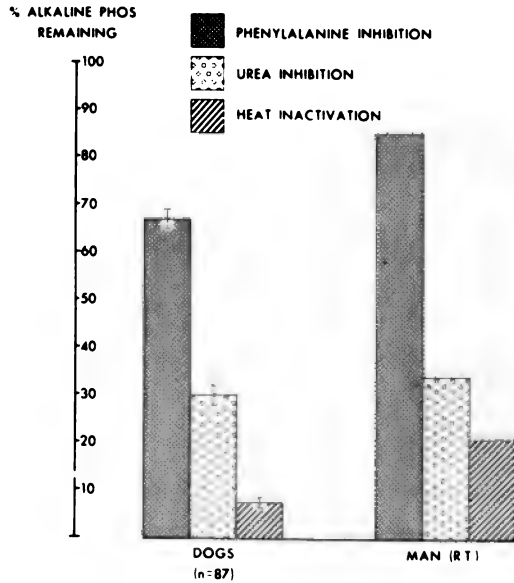
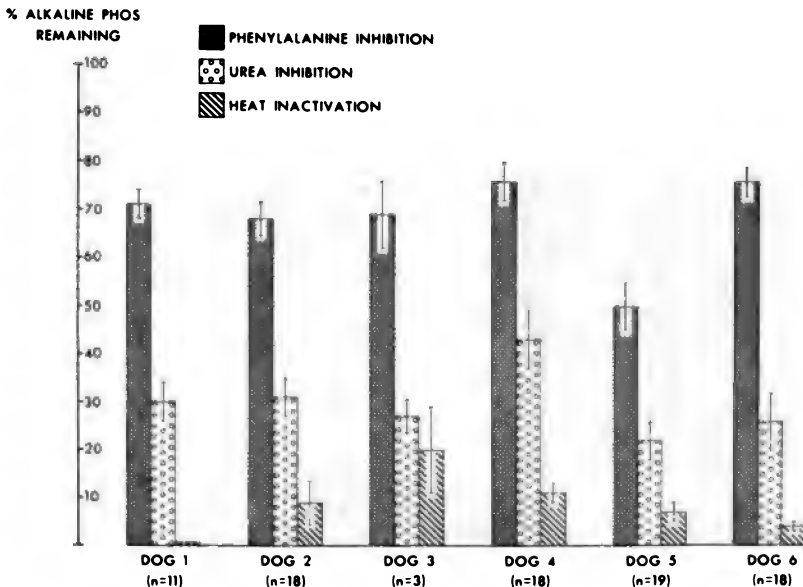


FIG. 2. Mean percent of alkaline phosphatase remaining in canine pancreatic juice and the percentage of enzyme remaining in human pancreatic fistula fluid after incubation with urea or L-phenylalanine or after heat inactivation. Bars at left represent mean  $\pm$  SEM of all collections from six dogs.



1. Percentage alkaline phosphatase remaining in pancreatic juice after incubation with urea or L-phenylalanine after heat inactivation. Each bar represents the mean  $\pm$  SEM of all 10-min collections in a single animal continuous intravenous infusion of secretin, 0.5 U/kg per hr. n = number of observations in each mean.

inhibition were 30% and 34%, respectively, and after heat inactivation were 7.5% and 21% respectively.

Figure 3 shows the electrophoretic mobility of the alkaline phosphatase isoenzyme in the human pancreatic fistula fluid compared to mobilities of isoenzymes derived from other human tissue sources. The pancreatic enzyme exhibited a pattern of mobility clearly different from that of any of the isoenzymes of other tissues sources.

**Discussion.** When Nothmann (1) found that ligation of the pancreatic duct in dogs resulted in a progressive increase in serum alkaline phosphatase activity, he assumed that this increased activity was of pancreatic origin. Subsequent studies (12, 13) have shown a significant increase in alkaline phosphatase concentration in duodenal juice after CCK-pancreozymin stimulation. The demonstration, by histochemical techniques, of this enzyme in various cellular components of the human pancreas (2) and the subsequent identification of distinctive isoenzyme characteristics of pancreatic alkaline phosphatase (3) are consistent with the presence of this enzyme in pancreatic secretory fluid.

Our data are in agreement with the findings of Warnes *et al.* (3) who showed that pancreatic alkaline phosphatase was much more sensitive to heat inactivation and urea inhibition than was the enzyme from the small intestine, but, in contrast, was largely unaffected by L-phenylalanine.

The question of whether increased serum

total alkaline phosphatase values 10 times reflect a predominant increase in pancreatic isoenzyme remains unanswered and must await isoenzyme characterization studies in subjects with acute inflammation as well as neoplastic disease of the pancreas. Additional techniques, such as acrylamide gel electrophoresis, will doubtless aid in refining our means of identifying the isoenzymes of different serum isoenzymes (14).

**Summary.** Alkaline phosphatase activity was measured in hormonally stimulated pancreatic juice from six dogs and in pancreatic fistula fluid from a human subject. Isoenzyme characterization studies, based on differences in susceptibilities to urea and L-phenylalanine inhibition and to heat inactivation, showed similarities between canine and human pancreatic secretory alkaline phosphatase compared to intestinal alkaline phosphatase. The pancreatic isoenzyme was much more sensitive to heat inactivation and urea inhibition but much more resistant to L-phenylalanine inhibition. The electrophoretic mobility of the enzyme present in human pancreatic fistula fluid was different from that of human serum, bone, or intestinal alkaline phosphatase.

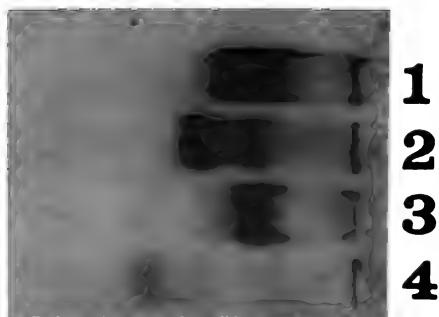


FIG. 3. Electrophoretic patterns (acrylamide gel) of alkaline phosphatase isoenzymes from human sources: 1, bone; 2, mixed liver and intestine; 3, intestine; and 4, pancreatic fistula fluid. Vertical electrophoresis in pH 9.0 Tris-malein acid buffer (0.283 M and 0.019 M, respectively) at 4°; 300 V for 3 hr; stained with sodium  $\alpha$ -naphthyl acid phosphate (1 hr).

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## Effect of Thymopoietin, Ubiquitin and Synthetic Serum Thymic Factor to Restore Immunocompetence in T-Cell Deficient Mice (40313)

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Role of the thymus in lymphocyte homeostasis (1, 2) and in conversion of precursors to thymus-dependent lymphocytes (T cells) has been the subject of investigation during the last 20 years. Atrophy of the thymus leads to diverse immunologic deficiencies that can be reversed by thymus grafts or by thymus grafts in cell-impermeable chambers (3-7), suggesting that the thymus might induce maturation of T lymphocytes through production of one or more factor(s). Candidate thymic factors have been prepared by several investigators. The biological activities of these factors have been assessed mainly by *in vitro* assays of T cell markers (e.g. thy-1 anti-lymphocyte populations (8-12), with no attention given to whether there is restoration of thymus-dependent immunizations measurable *in vivo*.

The present study was undertaken to evaluate several substances that induce T cell maturation for their ability to restore thymus-dependent immunocompetence in thymectomized mice. The substances tested were thymopoietin and ubiquitin, prepared by Dr. G. Goldstein (10, 11), and synthetic serum thymic factor, defined by Bach *et al.* (13).

**Materials and methods. Mice.** C58/J mice were obtained from the closed colony maintained at the Merck Sharp & Dohme Research Laboratories by Buckshire Farms, Perkasie, or were purchased from The Jackson Laboratories, Bar Harbor, Maine.

**Thymectomy and evaluation.** Partially purified thymopoietin (10) and ubiquitin (11) were prepared as aliquoted lyophilized preparations by Dr. Gideon Goldstein, Sloan-Kettering Research Center, New York. The substances to be tested were dissolved in phosphate-buffered saline (PBS) immediately before use. Mice were injected intraperitoneally

with the substances in 0.1 ml. Synthetic serum thymic factor (Pyroglu-Ala-Lys-Ser-Glu-Gly-Gly-Ser-Asn), defined by J-F Bach *et al.* (13), was synthesized by the peptide synthesis group (R. G. Strachan, W. J. Paleveda, S. J. Bergstrand, R. F. Nutt, R. Hirschmann, F. W. Holly, and D. F. Veber; manuscript in preparation), of Merck Sharp and Dohme Research Laboratories, and found to be positive for *in vitro* biological activity by Dr. J-F Bach, Necker Hôpital, Paris, France. Dosages, formulations and frequencies of treatment employed in these experiments were based on recommendations made by Dr. G. Goldstein for thymopoietin and ubiquitin, and by Dr. J-F Bach for serum thymic factor.

An unrelated pentapeptide, Asp-Ser-Asp-Pro-Arg (14), and a decapeptide, Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala (15), that failed to show *in vitro* biological activity in tests performed by Dr. J-F Bach were used for control purpose.

**Thymectomy.** Mice that were 0-2 days old were anesthetized by cooling at -20° (16) for 5-8 min (depending on size), thymectomized according to the method of Sjödin *et al.* (17), and then warmed under an infrared lamp (35°C) for 30 minutes. Young adult (4- to 6-week-old) mice were anesthetized by a single intraperitoneal injection (62.5 mg/kg) of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Illinois), and thymectomized according to the method of Dardenne and Bach (18). Sham thymectomized mice were treated surgically in the same manner except that the thymic lobes were not removed. At the appropriate time, all thymectomized mice were examined for presence of thymic remnants with the aid of a dissecting microscope. Mice found to have thymic remnants were excluded from the study.

**Anti-thymocyte serum treatment.** Heat-inactivated rabbit anti-mouse thymocyte and normal rabbit sera were purchased from Microbiological Associates, Bethesda, Maryland. A single 1 ml injection of serum was given intraperitoneally 3–4 days after thymectomy. Certain lots of anti-thymocyte sera were toxic for the mice and were not used.

**Preparation and administration of  $I_b$  cell suspensions.** The challenge inoculum was made by mixing equal volumes of viable ( $2 \times 10^3$ /ml) and irradiated ( $2 \times 10^6$ /ml) line  $I_b$  leukemic cell suspensions. Suspensions of viable (C58 mouse-derived)  $I_b$  cells were prepared in Hanks balanced salt solution as described previously (19). To prepare  $\gamma$ -irradiated  $I_b$  cells, suspensions of viable cells were exposed to 10,000R in a Model 109 Co<sup>60</sup> Irradiator (J. L. Shepherd and Assoc., Glendale, CA) that delivered 62,000R/min. Mice were injected intraperitoneally with 1 ml of the  $I_b$  cell mixture ( $10^3$  viable plus  $10^6$  irradiated cells). The mice were observed for 21 days and gross examination of the viscera of all mice that died was made to assure that deaths were due to leukemia.

**Mitogenic responses.** To test for capability to respond to mitogens, spleen and mesenteric lymph node cell suspensions were prepared in medium RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 5% fetal calf serum (Microbiological Associates). Five replicate cell suspensions, each containing  $4 \times 10^5$  cells in 0.2 ml, were prepared for testing the response to concanavalin A (Con A) (Miles Laboratories, Kankakee, IL) and phytohemagglutinin P (PHA) (Difco, Detroit, MI) in final concentration of 0.4  $\mu$ g/ml and 1:1000 dilution, respectively. After 3 days of incubation (37°, 5% CO<sub>2</sub>), 1  $\mu$ Ci of tritiated thymidine (New England Nuclear, Boston, MA) in 0.025 ml was added to each cell preparation, and incubation was continued for an additional 4 hr. The cells were harvested, washed to remove residual free fluids, and dissolved in 10 ml of Scintisol (Isolab, Akron, OH). The counts per minute were determined, and the mean cpm was calculated for the 5 replicate cultures in each group.

**Results. Failure of thymopoietin to restore T cell mitogen responses of lymphocytes from neonatally thymectomized C58 mice.** Findings

in preliminary experiments indicated both spleen and lymph node cells from treated or PBS-treated neonatally thymectomized C58 mice failed to be stimulated cell mitogens. In fact, incubation with Con A or PHA generally resulted in decreased <sup>3</sup>H-thymidine incorporation compared to thymectomized control cells that were not treated with antigen.

To test for ability of thymopoietin to restore T cell mitogen responses, neonatally thymectomized C58 mice were treated with thymopoietin for 4 weeks starting at 1 week of age. The animals were sacrificed by cervical dislocation 1 day following thymopoietin injection. Spleen and lymph node cells were removed from the animals and tested for mitogenic responses to Con A and PHA shown in Table I, treatment with thymopoietin did not restore normal responsiveness of the spleen and lymph node cells to the mitogens.

**Failure of thymopoietin and ubiquitin to restore resistance to line  $I_b$  leukemia in thymectomized C58 mice.** It was demonstrated in previous studies that normal adult mice develop an immune response (survival) when simultaneously vaccinated and challenged with a mixture of viable and line  $I_b$  leukemic cells, whereas immunosuppressed mice do not (19, 20). This immune response is highly dependent on functional maturity of the T-lymphocytes (21). A survival experiment was carried out in which adult thymectomized and sham thymectomized control animals were treated with rabbit anti-thymocyte serum to reduce the population of competent lymphocytes in the periphery. Animals were then challenged with the  $I_b$  cell preparation described above. As shown in Fig. 1, sham thymectomized animals initially highly susceptible to challenge  $I_b$  cells but their immunologic responsiveness was regained within 4 weeks after serum administration. By contrast, animals that had been thymectomized did not regain the resistance. Similar differences in regeneration of T cell mitogen responses (22) and in antibody bearing lymphocytes (23) were observed between adult thymectomized and sham thymectomized mice given anti-thymocyte serum.

An attempt was made to restore th

TABLE I. FAILURE OF THYMOPOIETIN TO RESTORE T CELL MITOGEN RESPONSIVENESS IN NEONATALLY THYMECTOMIZED C58 MICE.

In Vivo treatments <sup>a</sup>		In Vitro mitogen responses <sup>b</sup>					
		Spleen Cells			Lymph Node Cells		
		Control cpm (No. mice)	PHA stimulation index	Con A stimulation index	Control cpm (No. mice)	PHA stimulation index	Con A stimulation index
unoperated	None	3322 (5)	3.3	32.7	63 (6)	30	183
NTx	TP	4451 (8)	0.6	1.1	2878 (6)	0.1	0.5
NTx	PBS	4383 (2)	0.4	0.5	132 (2)	4.3	ND
NTx	None	4610 (8)	0.8	1.7	2677 (6)	0.7	0.4*

<sup>a</sup> Neonatally thymectomized (NTx) C58 mice were treated ip with 1  $\mu$ g thymopoietin (TP) or with PBS 5 $\times$ /week for 4 weeks (20 treatments) starting at 1 week of age.

<sup>b</sup> Averages of individual determination obtained from the indicated numbers of mice. Stimulation Index = Mitogen Stimulated cpm/control culture cpm

\* Data from two animals.

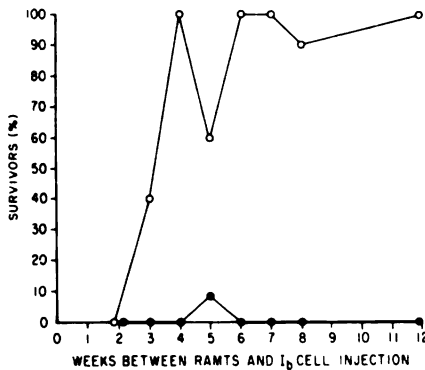


FIG. 1. Effect of adult thymectomy on recovery of the immune response to line I<sub>b</sub> leukemic cells following anti-thymocyte serum treatment. Groups of thymectomized (●) and sham thymectomized (○) mice were injected with an admixture of 10<sup>3</sup> viable I<sub>b</sub> cells and 10<sup>6</sup>  $\gamma$ -irradiated I<sub>b</sub> cells at the indicated times after the injection of 1 ml rabbit anti-mouse thymocyte serum. Each point represents 20 mice. Groups of thymectomized mice given normal rabbit serum survived the injection of I<sub>b</sub> cells.

munologic responsiveness of anti-thymocyte serum-treated adult thymectomized C58 mice by administration of thymopoietin or ubiquitin. Such mice were injected intraperitoneally with 1  $\mu$ g thymopoietin or ubiquitin 5 times per week for 5 weeks prior to challenge with I<sub>b</sub> cells. Neither thymopoietin nor ubiquitin restored the resistance of serum-treated thy-

mectomized animals to line I<sub>b</sub> leukemia (Table II). On the other hand, control serum-treated sham thymectomized mice were resistant to challenge.

*Failure of serum thymic factor to restore resistance to line I<sub>b</sub> leukemia in adult thymectomized C58 mice.* In similar experiments to those described above, anti-thymocyte serum-treated adult thymectomized C58 mice were injected 3 times per week for 8 weeks with synthetic serum thymic factor (0.1 ng) prepared with carboxymethylcellulose as described by M-A Bach (24). T cell immunocompetence was measured in terms of the survival rates of animals challenged with line I<sub>b</sub> leukemia. As shown in Table III, resistance to challenge was not restored to serum-treated thymectomized mice by treatment with serum thymic factor. Control animals that had been sham thymectomized and treated with anti-thymocyte serum were resistant to challenge.

In the experiments just described, repeated injections of carboxymethylcellulose, in which the test preparations were suspended, was toxic; causing skin nodules, ulceration and death in roughly half the animals during the 8 week period of treatment. To avoid this, adult thymectomized animals that had been given anti-thymocyte serum were treated 5 times per week for 8 weeks with 1  $\mu$ g serum thymic factor in PBS and then challenged



TABLE II. LACK OF EFFECT OF THYMOPOIETIN AND UBIQUITIN ON RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I<sub>b</sub> LEUKEMIA.

Treatment of mice		Response to line I <sub>b</sub> leukemia <sup>c</sup>	
Pre-Therapy T Cell Depletion <sup>a</sup>	Substance Tested <sup>b</sup>	No. of surviving/total (%)	Average time of death in days (± SD)
<i>Thymectomized test animals</i>			
ATx, RAMTS	Thymopoietin	0/16 (0)	10.69 ± 0.60
ATx, RAMTS	Ubiquitin	1/13 (8)	10.69 ± 0.78
<i>Thymectomized control animals</i>			
ATx, RAMTS	PBS	0/12 (0)	10.75 ± 1.06
ATx, RAMTS	None	0/13 (0)	10.92 ± 1.75
<i>Nonthymectomized control animals</i>			
STx, RAMTS	PBS	11/12 (92)	*
Unoperated	PBS	20/20 (100)	

<sup>a</sup> C58 mice were adult thymectomized (ATx) or sham operated (STx) and given rabbit anti-mouse thymocyte serum (RAMTS) 3 days later.

<sup>b</sup> Treated mice received 1 µg of thymopoietin or ubiquitin ip 5×/week for 5 weeks.

<sup>c</sup> All mice were challenged with a mixture of 10<sup>3</sup> viable and 10<sup>6</sup> irradiated I<sub>b</sub> cells.

\* One mouse died on day 15.

TABLE III. LACK OF EFFECT OF COMPLEXED SERUM THYMIC FACTOR ON THE RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I<sub>b</sub> LEUKEMIA.

Treatment of mice		Deaths following treatment with the test substances in CMC			Survival following challenge with line I <sub>b</sub> leukemia (%)
Pre-therapy T cell depletion <sup>a</sup>	Substance tested <sup>b</sup>	No. of mice		Survival (%)	
		Start	Final		
<i>Thymectomized test animals</i>					
ATx, RAMTS	Serum thymic factor	15	7	(46)	1/7 (14)
ATx, RAMTS	Decapeptide	15	12	(80)	4/11 (36)
<i>Thymectomized control animals</i>					
Atx, RAMTS	Buffered saline solution	15	8	(53)	0/7 (0)
<i>Sham thymectomized control animals</i>					
STx, RAMTS	Buffered saline solution	15	9	(60)	9/9 (100)

<sup>a</sup> C58 mice were adult thymectomized (ATx) or sham operated (STx) and given rabbit anti-mouse thymocyte serum (RAMTS) four days later.

<sup>b</sup> All test substances were contained in carboxymethylcellulose (CMC) that was highly toxic, causing deaths in the animals. Treated mice received 0.5 ml sc containing 0.1 ng of serum thymic factor or control decapeptide combined with 27 mg CMC. Treatment was started 6 days following RAMTS treatment and continued three times per week for a total of 20 injections. CMC and total volume of treatment were reduced to 5 mg and 0.1 ml, respectively, after seven injections.

with I<sub>b</sub> cells. The findings given in Table IV show that the thymic factor in PBS, as in carboxymethylcellulose, failed to restore immunocompetence to the mice.

**Discussion.** The main criterion used to classify materials as thymic hormones has been their capacity to induce T cell surface membrane markers on lymphocytes. However, the induction of such cell markers seems not to reflect a maturation event specifically induced by thymic hormone, since many unrelated substances, including nonthymic tis-

sue extracts, ubiquitin, poly A:U, endotoxin, prolactin, glucagon, prostaglandin E and histamine, all have the ability to induce the same cell surface markers (11, 25, 26). Therefore, it is of value to test candidate thymic hormones in more discriminating assays; assays that would measure the effect on immunocompetence. The present studies were carried out to measure the ability, if any, of thymopoietin, ubiquitin, and serum thymic factor to restore immunocompetence *in vivo* in T lymphocyte deficient C58 mice. Daily admin-

TABLE IV. LACK OF EFFECT OF UNCOMPLEXED SERUM THYMIC FACTOR ON THE RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I<sub>b</sub> LEUKEMIA.

Treatment of Mice <sup>a</sup>		Deaths following treatment with the test substances			Survival following challenge with line I <sub>b</sub> leukemia (%)
Group	Substance tested	No. of mice		Survival (%)	
		Start	Final		
<i>Thymectomized test animals</i>					
TS	Serum thymic factor	13	9	(70)	2/9 (22)
TS	Pentapeptide	13	12	(92)	3/12 (25)
<i>Thymectomized control animals</i>					
TS	Buffered saline solution	13	13	(100)	4/13 (31)
<i>Sham thymectomized control animals</i>					
TS	Buffered saline solution	13	12	(92)	12/12 (100)

<sup>a</sup> Mice were treated as indicated in Table III except that each mouse received 0.1 ml PBS sc containing 1 ng of thymic factor or control pentapeptide five times per week for a total of 36 injections.

of thymopoietin to neonatally thymectomized C58 mice for 4 weeks did not alter the responsiveness of their lymph node spleen cells to T cell mitogens. Daily treatments of anti-thymocyte serum-treated adult thymectomized C58 mice with thymopoietin, ubiquitin, or serum thymic factor were ineffective in restoring the ability of these mice to resist challenge with line I<sub>b</sub> leukemia. By contrast, sham thymectomized C58 mice, that had received anti-thymocyte serum, recovered their resistance to cell challenge spontaneously within 2 weeks after serum treatment. Thus, thymopoietin and serum thymic factor, in the doses used, did not mimic the restorative effect provided by the intact thymus.

Failure of the candidate thymic factors under investigation to restore immunocompetence in T cell deficient mice might be due to insufficient exposure of the precursors to these substances or to total irrelevance of the substances to the cell maturation process. The more likely explanation, as suggested by A. L. Goldstein *et al.*, is that immunologic maturation is a process involving a number of steps and that thymic factor, initiating a single cellular step, might not be reflected in any meaningful immunologic activity. Scheid *et al.*, for example, have demonstrated that *in vivo* (28) and (26) treatment with thymopoietin did not alter TL and thy-1 antigens on murine lymphocytes. However, lymphocytes that lack the TL marker are known to be immunoincompetent (29). Similarly, Miller *et al.* (8, 24) have shown that serum

thymic factor can maintain a normal level of short-lived thy-1-positive lymphocytes in adult thymectomized mice. Yet, the responses of adult C58 mice to T cell mitogens and line I<sub>b</sub> leukemia are dependent on a long-lived population of thy-1-positive lymphocytes. Perhaps the evaluation of only one substance at a time would inevitably result in failure to induce immunocompetence.

**Abstract.** Thymopoietin, ubiquitin, and serum thymic factor, all of which induce T cell markers on lymphocytes, have been evaluated for their capacity to induce thymus-dependent activities *in vivo*. Multiple treatments over a period of weeks failed to restore either resistance to line I<sub>b</sub> leukemia or responses to T cell mitogens in T cell-deficient C58 mice. The findings suggest that these substances are ineffective in inducing thymus-dependent immunocompetence that is meaningful in the intact animal.

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## Inactivated Hepatitis A Virus Vaccine Prepared from Infected Marmoset Liver (40314)

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olation of the CR326 strain of human

A virus in mystax marmosets was from these laboratories (1, 2) in 1973 virus was shown to be inactivated by hyde (3). It was demonstrated, suby, that the livers of white-mousnd rufiventer marmosets (*S. mystax labiatus*, respectively) infected with irus contained large amounts of hep-iral antigen (3-6). This made possi-development of the first practical r hepatitis A virus antigen and anti-the complement fixation and im-herence (IA) methods. The present howed that CR326 strain hepatitis A ccine, purified from infected mar-ver and inactivated with formalde-duced homologous IA antibody and 1 marmosets against hepatitis A virus e.

*Materials and methods. Marmosets.* Wild-rufiventer (*S. labiatus*) marmosets d. The animals were conditioned and ed as described previously (1).

t. Isocitric dehydrogenase (ICD) as-e performed, as previously described the marmoset plasmas collected at ntervals. Values of 1500 Sigma units r obtained for two or more consec-eks were considered to be indicative ion. Assays for hepatitis A antibody armoset sera were by the IA proce-cribed earlier (5).

*Hepatitis A vaccine.* A rufiventer marmoset ted intravenously with 25th rufiven- oset passage CR326 hepatitis A vi-liver was perfused *in situ* with phos-ffered saline solution (PBS) and re-rom the marmoset at the time that noset first showed pronounced ICD on the 14th day after inoculation. ted liver was homogenized with PBS mortar and pestle with alundum to 0% suspension. The supernate was

collected following low speed centrifugation and was diluted further to give a 5% liver extract. The extract was then heated at 60° for 30 min after which it was further clarified by centrifugation at 2500 rpm for 30 min yielding an amber-colored supernate that was slightly opalescent. Formalin in a final concentration of 1:4000 was added to the super-nate, and the mixture was incubated with continuing agitation at 35.5° for 4 days. The formalin was then partially neutralized with sodium bisulfite to give a final concentration of 10 µg/ml formaldehyde. This was the vac-cine, and it was stored at 4°. The viral particle content per ml was  $1.4 \times 10^{10}$  as measured by electron microscopy and the hepatitis A antigen titer was 1:8 by IA. The liver from a noninfected rufiventer marmoset was processed in an identical way to produce vaccine for control purpose.

*Vaccination.* Rufiventer marmosets were employed, and all were initially devoid of human hepatitis A virus antibody. Eight mar-mosets were each given 1 ml amounts of hepatitis A vaccine subcutaneously at bi-weekly intervals for 14 weeks (eight injec-tions). An additional eight animals were injected subcutaneously at the same time with normal marmoset liver vaccine. Six more animals were each given an intravenous injection of 1 ml of hepatitis A vaccine on a single occasion for testing for absence of live hepa-titis A virus in the vaccine.

*Marmoset challenge.* All marmosets were challenged intravenously 17 weeks after the first vaccine injection, with 1 ml of a  $10^{-6}$  dilution of CR326 hepatitis A virus contain-ing approximately  $10^3$  fifty percent marmoset infectious doses of virus.

*Results. IA antibody responses.* Serum anti-body titrations were performed on plasma samples collected at weekly intervals during the 17-week immunization regimen and the 9-week period following challenge. Figure 1

shows that all eight animals displayed IA antibody after the sixth vaccine dose had been given (by 12 weeks), at least three of the animals having responded after the fifth dose. The titers ranged from 1:20 to 1:320. None of the animals given control vaccine developed hepatitis A antibody. One of the six animals in the viral inactivation test group that received a single dose of vaccine intravenously developed antibody by the 12th week after injection. Table I shows that none of the animals in any group developed positive ICD enzyme elevations prior to challenge indicating that the materials given to the animals did not contain live hepatitis A virus.

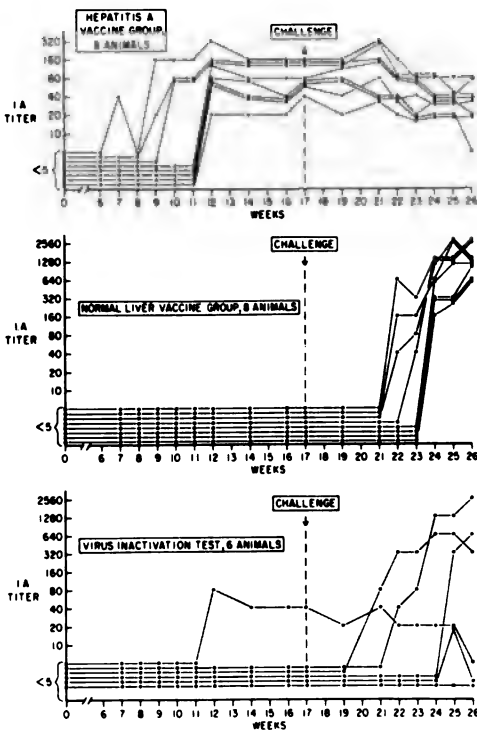


FIG. 1. Hepatitis A antibody responses in rufiventer marmosets as measured by immune adherence during the immunization and challenge regimens.

**Protective efficacy.** The marmosets in all three groups were challenged intravenously with live CR326 hepatitis A virus during the 17th week after vaccination was initiated, and the findings are shown in Fig. 1 and Table I. All eight of the marmosets that received the normal liver vaccine showed elevations in ICD and all developed IA antibody with titers ranging from 1:640 to 1:2560 or greater. By contrast, none of the animals given hepatitis A vaccine showed ICD elevations and none showed more than twofold increase in antibody titer. Interestingly, only two of the six animals that were given a single dose of vaccine intravenously showed elevations in ICD, and these two animals developed IA antibody. One other animal developed pronounced IA antibody, without an ICD elevation. All of the three remaining animals appeared to be protected even though only one had IA antibody prior to challenge. These findings indicated that the vaccine given subcutaneously in multiple injections was highly effective in preventing experimental hepatitis on challenge in marmosets and that even a single dose of vaccine given intravenously afforded protection to live virus challenge in some animals.

**Discussion.** The work on which the present findings are based represents the first demonstration that inactivated hepatitis A virus can afford protection against live hepatitis A virus challenge. Vaccine was given in eight divided aqueous doses, and it seems likely that protection might have been afforded following fewer doses, especially if an immunologic adjuvant had been employed. This vaccine might prove equally effective in preventing hepatitis A in man and might, therefore, be of extreme importance in the control of the disease. The limited availability of marmosets and the lack of ability, to date, to achieve practical replication of the virus in

TABLE I. ANTIBODY AND ENZYME DETERMINATIONS IN A CONTROLLED STUDY OF HUMAN HEPATITIS A VACCINE IN MARMOSETS.

Marmoset group	Time period				Protective efficacy of vaccine
	Before hepatitis A virus challenge		After hepatitis A virus challenge		
	Antibody response No. Pos./Total	Enzyme elevation No. Pos./Total	Antibody response No. Pos./Total	Enzyme elevation No. Pos./Total	
Normal liver vaccine	0/8	0/8	8/8	8/8	100% (partial protection by i.v. vaccine administration)
Hepatitis A vaccine	8/8	0/8	0/8	0/8	
Virus inactivation test	1/6	0/6	3/6	2/6	

the laboratory precludes any substantial progress toward routine immunization in man at the present time.

**Summary.** Human hepatitis A virus, partially purified from the liver of a rufiventer marmoset infected with CR326 strain virus, was inactivated with formalin and was shown to be highly potent in stimulating homologous antibody in marmosets when administered subcutaneously at bi-weekly intervals in eight divided doses. The vaccine was shown to prevent hepatitis A in all marmosets when challenged with live hepatitis A virus in a controlled study.

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# Structural Determinants of the Renal Tubular Activity of Vitamin D<sub>3</sub> Derivatives: with 1 $\alpha$ -Hydroxy, 24*R*,25-Dihydroxy, and 1 $\alpha$ ,24*R*,25-Trihydroxy Vitamin D<sub>3</sub> (

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Previous studies from this and other laboratories have documented an acute effect of vitamin D<sub>3</sub> and its major metabolites on renal tubular electrolyte transport (1, 2). The infusion of the biologically active metabolites of vitamin D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> (25-hydroxycholecalciferol, 25-HCC) and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-dihydroxycholecalciferol, 1,25-DHCC), have been shown to produce an enhancement of phosphate, calcium, and sodium reabsorption both in the dog and rat (1-6). Recently, substantial progress has been made in the identification and biochemical synthesis of other naturally occurring vitamin D metabolites as well as structural analogs. These advancements have provided us with the opportunity to study the renal tubular effects of additional metabolites and analogs of the parent compound and to identify the structural requirements of these vitamin D<sub>3</sub> derivatives with regard to their transport actions. The data demonstrate that in order for an antiphosphaturia to occur, the derivative must contain a hydroxyl group in the 1 position. Furthermore, no effect on calcium or sodium transport is evident unless the compound possesses a 25-hydroxyl group which is sterically unhindered.

**Methods.** Acute clearance studies were performed in female mongrel dogs weighing 16 to 23 kg which had been thyroparathyroidectomized (TPTX) at least 48 hr prior to the experiment. Details of the surgical procedures and clearance technique have been reported elsewhere (1, 3). Completeness of parathyroidectomy was verified by comparing the serum calcium concentration 2 to 4 days postoperatively to those just before the procedure. Animals with at least a 30% reduction in serum calcium were selected for study. Thyroid replacement was accomplished by oral administration of 0.1 to 0.2 mg of synthroid (Flint) daily. The animals were fasted and thirsted for 16 hr before the study and re-

ceived 5 U of vasopressin (Pitressin in oil, Parke, Davis and Company) prior to the study. The dogs were anesthetized with 25 mg/kg of sodium pentobarbital with subsequent intermittent doses as required. A cuffed tracheal tube was inserted and the animal ventilated with a Harvard respirator. Catheters were inserted into a hindlimb vein for infusion of saline and into the external jugular vein for blood sampling. Priming doses of inulin and *p*-aminohippurate (PAH) were administered and a sustaining infusion of these substances was administered at a rate of 1 ml/min in physiological saline. Aqueous vasopressin was added to the saline infusion in an amount calculated to effect a rate of 0.5 mU/min. Volume expansion was produced by infusing a 0.9% saline solution containing 1.0 to 1.5 mEq/liter of calcium chloride. The total amount of saline infused was approximately 2.5% of animal body weight, after which urinary losses were replaced by adjusting the rate of infusion so that volume expansion was sustained. Urine collections were begun and after a steady state was achieved, one of the following experimental maneuvers was performed. In Group A (group A, control animals), 0.25 ml of vehicle (propylene glycol) was injected intravenously as a bolus. The experiment continued for approximately 2 hr, during which 10 to 12 clearance periods of approximately 10 min each were obtained. In Group B (other dogs (group B) 0.625  $\mu$ g of 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> (1 $\alpha$ -hydroxycholecalciferol, HCC)<sup>1</sup> dissolved in 0.25 ml of propylene glycol was given according to the same protocol as described for the control group. Group C consisted of five dogs which

<sup>1</sup> The 1 $\alpha$ -HCC utilized in this study was supplied by Dr. Jack Hinman, Upjohn Company, Kalamazoo, Mich.

0.625  $\mu$ g of 24R,25-dihydroxyvitamin (24R,25-dihydroxycholecalciferol, 24R,25-DHCC)<sup>2</sup> dissolved in 0.25 ml of propylene glycol. The animals in experimental group D received 0.625  $\mu$ g of 1 $\alpha$ ,24R,25-THCC dissolved in 0.25 ml of propylene glycol. Blood was drawn at the beginning of the study, at the plateau of each steady state, before each experimental maneuver, and every 30 min throughout the study. Blood and urine were analyzed for inulin, PAH, phosphorus, calcium, and sodium by methods previously described from this laboratory (1). Serum ultrafiltrates were obtained by centrifuging sealed anaerobically through CF-50 ultrafilter cones (Amicon Corp., Lexington, Mass.). Statistical evaluation of the data was performed by paired *t* test.

**Results.** Table I summarizes the data obtained in the control experiments (group A) as well as those in which the synthetic analog of vitamin D, 1 $\alpha$ -HCC (group B) or the vitamin D metabolites, 24R,25-DHCC (group C) or 1 $\alpha$ ,24R,25-THCC (group D), were administered intravenously. In the control animals the intravenous administration of propylene glycol did not cause any changes either in the absolute urinary excretion of ions or in their percentage excretion (Figs. 1–3, group A, Table I). Neither renal hemodynamics nor serum ultrafilterable calcium concentration (SUF<sub>Ca</sub>) altered in any consistent manner. The acute administration of 0.625  $\mu$ g of 24R,25-DHCC had no effect on either the absolute or percentage excretion rates of phosphate, calcium, or sodium (group C, Table I). The mean delta  $\Delta$  was  $-3.6 \pm 3.9\%$  ( $P > 0.40$ ). However, 1 $\alpha$ ,24R,25-THCC and 1 $\alpha$ -HCC, when given in the same amount (0.625  $\mu$ g), induced a significant decline (by 28 and 30%, respectively) in the percentage excretion of phosphate ( $P < 0.01$ ,  $< 0.05$ ). The mean changes in phosphate excretion were  $-6.6 \pm 1.6$  and  $-5.1 \pm 1.4\%$ , respectively. These decrements were accompanied by reductions in the absolute excretion rates of phosphate of 18 and 20%, respectively, which were also statistically significant ( $P < 0.05$ ,  $< 0.02$ , Fig. 1). There was no significant change in either absolute or

percentage calcium or sodium excretion was observed in the animals receiving 1 $\alpha$ ,24R,25-THCC and 1 $\alpha$ -HCC (groups C and D, Table I, Figs. 2 and 3). Glomerular filtration rate and effective renal plasma flow as measured by the clearances of inulin and PAH, respectively, were unaltered. There was no statistically significant change in the level of serum sodium concentration in any of the groups. SUF<sub>Ca</sub> decreased slightly but consistently in group C (from  $1.76 \pm 0.08$  to  $1.70 \pm 0.07$  mmole/liter) after 24R,25-DHCC was administered. This change did not affect either filtered load or excretion rate. In all of the other groups there was no significant change in either SUF<sub>Ca</sub> or SUF phosphate.

**Discussion.** Previous studies of the biological activities of 24R,25-DHCC and 1 $\alpha$ ,24R,25-THCC have been limited to an evaluation of these substances in the skeleton and gastrointestinal tract. Furthermore, not only is there only a single study of the effects of 1 $\alpha$ -HCC on the kidney (8) but none of the studies involving these compounds have been performed in the dog. The vitamin D<sub>3</sub> derivatives utilized in this study were evaluated, therefore, with the following objectives in mind. First, it was our intention to attempt to establish their respective capacities to alter renal electrolyte transport and to compare these experimental observations with those previously obtained with 25-HCC and 1,25-DHCC (1, 2). Second, the availability of these agents provided us with the opportunity to investigate what might be the structural determinants of vitamin D metabolites as regards their ability to alter electrolyte reabsorption at the renal tubular level.

These newly described derivatives of the parent vitamin have recently been shown to have substantial activity in stimulating intestinal calcium and phosphate transport (9–12). They are also active in elevating serum calcium and phosphorus and in the mobilization of calcium from bone in rachitic rats (12). However, in the latter systems, the response to 24R,25-DHCC occurs after a considerable time lag (13). Furthermore, nephrectomy and a high calcium diet abolished the effect of 24R,25-DHCC on intestinal calcium transport (9, 13). This finding suggests that renal conversion of this metabolite to 1 $\alpha$ ,24R,25-THCC or some other more polar metabolite

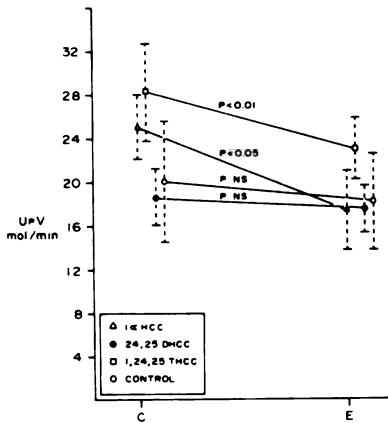
<sup>2</sup> Kindly provided by Dr. Milan Uskokovic, Roche Laboratories, Nutley, N.J.



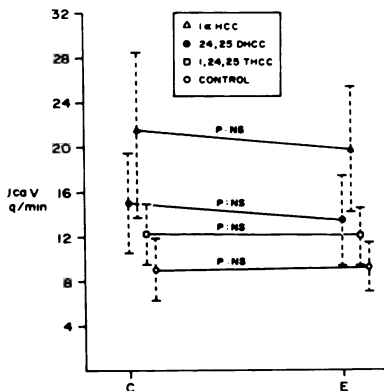
TABLE I. EFFECT OF VITAMIN D METABOLITES ON ELECTROLYTE EXCRETION AND RENAL HEMODYNAMICS.<sup>a</sup>

	A (N = 5) Control		B (N = 5) 1 $\alpha$ -HCC		C (N = 5) 24R,25-DHCC		D (N = 7) 1,24R,25-THCC	
	C	E	C	E	C	E	C	E
C <sub>IN</sub> (ml/min)	49.7 $\pm$ 6.0	47.5 $\pm$ 5.0	86.4 $\pm$ 5.6	87.8 $\pm$ 6.5	71.44 $\pm$ 1.6	67.5 $\pm$ 3.5	63.96 $\pm$ 7.6	69.4 $\pm$ 6.1
	P = NS		P = NS		P = NS		P = NS	
C <sub>PAH</sub> (ml/min)	133.6 $\pm$ 22.5	138.0 $\pm$ 20.8	263.6 $\pm$ 34.6	269.0 $\pm$ 32.5	273.7 $\pm$ 32.6	258.3 $\pm$ 23.6	146.7 $\pm$ 20.5	122.8 $\pm$ 22.5
	P = NS		P = NS		P = NS		P = NS	
%E <sub>PO<sub>4</sub></sub>	19.4 $\pm$ 4.0	20.3 $\pm$ 4.2	15.6 $\pm$ 2.4	10.9 $\pm$ 2.5	20.5 $\pm$ 3.7	16.8 $\pm$ 3.0	23.9 $\pm$ 3.3	17.3 $\pm$ 2.3
	P = NS		P < 0.05		P = NS		P < 0.01	
%E <sub>Ca</sub>	8.4 $\pm$ 2.0	8.9 $\pm$ 1.3	13.4 $\pm$ 4.3	12.1 $\pm$ 3.6	10.5 $\pm$ 2.6	10.0 $\pm$ 2.1	10.6 $\pm$ 1.9	9.9 $\pm$ 1.8
	P = NS		P = NS		P = NS		P = NS	
%E <sub>Na</sub>	9.0 $\pm$ 2.2	10.6 $\pm$ 1.6	14.0 $\pm$ 4.0	13.7 $\pm$ 3.6	12.8 $\pm$ 2.7	13.0 $\pm$ 2.4	10.9 $\pm$ 1.5	10.5 $\pm$ 1.5
	P = NS		P = NS		P = NS		P = NS	
SUF <sub>Ca</sub> ( $\mu$ Eq/liter)	2.03 $\pm$ 0.05	2.08 $\pm$ 0.02	1.87 $\pm$ 0.13	1.89 $\pm$ 0.15	1.81 $\pm$ 0.16	1.79 $\pm$ 0.17	1.76 $\pm$ 0.07	1.70 $\pm$ 0.07
	P = NS		P = NS		P = NS		P < 0.05	

<sup>a</sup> Abbreviations: 1 $\alpha$ -HCC, 1 $\alpha$ -hydroxyvitamin D<sub>3</sub>; 24R,25-DHCC, 24R,25-dihydroxyvitamin D<sub>3</sub>; 1 $\alpha$ ,24R,25-THCC, 1 $\alpha$ ,24R,25-trihydroxycholecalciferol. N, number of studies. C and E, control and experimental phases of the experiment, respectively. C<sub>IN</sub> and C<sub>PAH</sub>, glomerular filtration rate and effective renal plasma flow as estimated by the clearances of inulin and p-aminohippurate, respectively. %E<sub>P</sub>, %E<sub>Ca</sub>, and %E<sub>Na</sub>, % excretion rates of phosphate, calcium, and sodium, respectively. SUF<sub>Ca</sub>, serum ultrafilterable calcium concentration.



1. The effects of vitamin D<sub>3</sub> metabolites and 1α on the absolute excretion rate of phosphate. Note significant reduction in phosphate excretion following administration of both 1α-HCC and 1α,24R,25-24R,25-HCC was without effect on urinary phosphorexcretion. Data points represent the mean values for all dogs before (C) and after (E) administration of compounds.



2. The effect of vitamin D<sub>3</sub> metabolites and 1α on urinary calcium excretion. All three compounds, DHCC, 1α,24R,25-THCC, and 1α-HCC, were without effect on the renal tubular handling of calcium.

required for its biological activity to be demonstrated (13). Indeed, radioactive 24R,25-DHCC has been shown to be further metabolized *in vivo* as well as *in vitro* to 1α,24R,25-THCC (14). It seems likely that hydroxylation at the 1 position is a prerequisite step in the metabolism of 24R,25-DHCC for this compound to become biologically active.

Results on renal phosphate excretion and the observations obtained in the in vivo transport system that hydroxylation

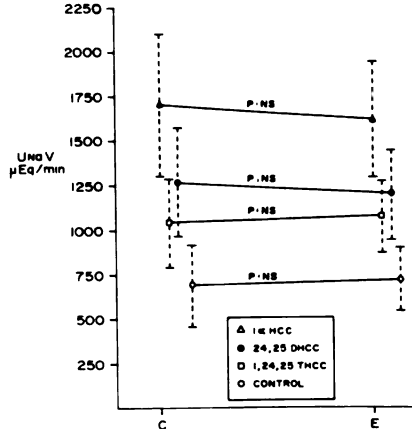


FIG. 3. The effects of vitamin D<sub>3</sub> metabolites on the urinary excretion of sodium. Note that none of the agents (1α-HCC, 24R,25-DHCC, or 1α,24R,25-THCC) changed urinary sodium excretion.

at the 1 position is necessary for the manifestation of its biological activity. Thus, a common feature of all the compounds which in the present study effected a reduction in phosphate excretion, was the presence of the 1α-hydroxy configuration. Since glomerular filtration rate and effective renal plasma flow, as well as the filtered load of phosphorus were unchanged (Table I), it is reasonable to conclude that the changes observed were due to a direct action of these compounds on the renal tubule. Furthermore, we conclude from these findings that the action of vitamin D metabolites on the tubular transport of phosphate depends upon the presence of the 1α-hydroxyl configuration. Since, in earlier studies from this laboratory, 25-HCC was likewise very effective in reducing phosphate excretion (1), we infer that the latter metabolite was converted *in vivo* to another vitamin D<sub>3</sub> derivative containing a 1α-hydroxyl group. This most probably means the formation of 1α,25-DHCC or some other "tissue active" substance, as yet unidentified (2). The fact that 25-HCC does not act immediately on renal electrolyte transport (1) and requires the "permissive" effect of either parathyroid hormone (4, 7) or vasopressin (6) for its renal tubular effects to become evident, are supportive of this thesis.

Perhaps the most important synthetic analog of 1α,25-DHCC currently available, at least from a therapeutic standpoint, is 1α-

HCC. This compound is almost as potent as 1 $\alpha$ ,25-DHCC in stimulating intestinal calcium transport in the chicken (15) and has approximately two to five times the activity of vitamin D<sub>3</sub> on calcification of the skeleton and in stimulating gut absorption of calcium in the rat (16). It has recently been reported that the intravenous administration of 1 $\alpha$ -HCC to the rachitic rat produces an enhancement of intestinal calcium absorption within 1 hr of its infusion (17). This extremely rapid onset of action suggested to the investigators that 1 $\alpha$ -HCC might act directly on the cell membrane transport of calcium. However, studies by Zerwekh *et al.* (18) suggest that the action of 1 $\alpha$ -HCC requires prior conversion to 1 $\alpha$ ,25-DHCC. In addition, it has been demonstrated that tritiated 1 $\alpha$ ,25-DHCC appears in the intestine and bone within 2 hr after intravenous administration of 1 $\alpha$ -[6-<sup>3</sup>H]hydroxy vitamin D<sub>3</sub> (19). The design of the acute clearance studies presented in this report was such that observations were made for only 2 hr following the administration of the vitamin D<sub>3</sub> derivatives. Thus, since further metabolic conversion of both 1 $\alpha$ -HCC and 24R,25-DHCC appears to require longer than 2 hr, we presume that the observed changes in renal transport were due to the action of the unchanged compounds. As regards the effects of 1 $\alpha$ -HCC, our results confirm the observations of Pechet and Hesse (8) and Toffolon *et al.* (17) that 1 $\alpha$ -HCC has a very rapid onset of action. Of course, we cannot rule out the possibility of some (more rapid) metabolism of these substances to an as yet unidentified "tissue-active" metabolite or metabolites.

Unlike 25-HCC and 1,25-DHCC (1, 2) none of the vitamin D<sub>3</sub> derivatives examined in the current study (1 $\alpha$ -HCC, 24R,25-DHCC, or 1 $\alpha$ ,24R,25-THCC) were effective in altering either sodium or calcium excretion when given acutely. While no explanation of these observations is conclusively provided by the data, the findings could be explained as follows. The 1 $\alpha$ -hydroxylated compounds (1 $\alpha$ -HCC and 1 $\alpha$ ,24R,25-THCC) may act at different sites within the nephron or other receptor molecules than those affected by 25-HCC and 1 $\alpha$ ,25-DHCC. Alternatively, it may be that in order for a compound to alter calcium and sodium reabsorption, it must

have a hydroxyl group in the 25 position in both the 1 and 25 positions. Further, it appears that the 25-hydroxyl group also be sterically unhindered. Indeed, *et al.* recently presented evidence that 25-HCC was rather less potent than 1 $\alpha$ -HCC or 24R-HCC in its ability to effect resorption (20). They proposed that steric factors, steric hindrance, or an excess of hydrophilic groups in this region may explain the decreased activity of this compound. Further study will be required to elucidate the mechanism of the above postulated mechanisms (20).

**Summary.** The acute effects of DHCC, 1 $\alpha$ ,24R,25-THCC, and 1 $\alpha$ -HCC on the renal handling of phosphate, calcium, and sodium were evaluated in the TF<sub>1</sub> rat which had been mildly volume expanded and infused with vasopressin to establish polyuria. Both 1 $\alpha$ -HCC and 1 $\alpha$ ,24R,25-THCC when given intravenously in a dose of 0.625  $\mu$ g produced a significant decrease in urinary phosphate excretion. Phosphate excretion decreased by 28%, respectively ( $P < 0.05$ ,  $P < 0.01$ ). There was no alteration in renal hemodynamics or in the filtered load of this ion. These results suggest a direct action of these compounds on renal tubular transport mechanisms. No change in the urinary excretion of calcium or sodium was observed following administration of the two vitamin D<sub>3</sub> derivatives. 24R,25-DHCC was without effect on the renal handling of all three ions.

When previous experimental findings regarding the renal actions of 25-HCC and 1 $\alpha$ ,25-DHCC are considered, the data suggest that the 1-hydroxyl grouping is required for the metabolites of vitamin D to effect changes in phosphate transport at the renal tubule. It appears that a sterically unhindered hydroxyl group is necessary in order for vitamin D<sub>3</sub> derivatives to act on the renal tubule for regulation of either calcium or sodium.

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# Stimulation of Growth Hormone Release by Intraventricular Administration of 5HT or Quipazine in Unanesthetized Male Rats<sup>1</sup> (40316)

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Although there is considerable evidence in favor of a stimulating role of the central serotonergic system on the secretion of GH in man and nonhuman primates (1-4), little information is available in other species (5). Collu *et al.* (6) reported that intraventricular injection of serotonin stimulated GH secretion in rats anesthetized with urethane and that the effect was abolished by phenoxybenzamine, an  $\alpha$  receptor blocker. Since experiments on animals in deep urethane anesthesia are opened to some criticism, we have investigated, in the present work, the effect of intraventricular administration of serotonin in unanesthetized unrestrained rats and compared them with the effect of intraventricular administration of the serotonin receptor agonist quipazine (7).

**Materials and Methods.** Adult male rats of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, California) were used. They were housed under controlled conditions of lighting (light on from 0500 to 1900 hr) and temperature ( $24 \pm 1^\circ$ ) with free access to food and water. After 2 weeks of adaptation in our animal facility, a 23-gauge stainless-steel cannula was implanted into the third ventricle and 1 week later the animals were fitted with Silastic intravenous catheters as described earlier (8, 9). On the day of the experiment, usually 2 days after implantation of the intravenous cannulas, the animals were transferred in their cages into a quiet laboratory and polyethylene extension tubes (PE50, 12 in. long) filled with a solution of heparin in 0.9% NaCl were attached to the distal end of permanent iv cannulas. Thirty to sixty minutes later a preinjection blood sample (0.6-0.8 ml) was withdrawn; then the intraventricular injection was performed and postinjection samples (0.6-0.8 ml) were taken

at 10, 30, and 60 min into heparinized syringes. The volume of each sample was replaced immediately after each bleeding by an equal volume of 0.9% saline.

The intraventricular injections were performed according to the procedure described earlier (8, 9): Serotonin (serotonin creatinine sulfate complex, Calbiochem) or quipazine maleate (gift of Miles Labs, Inc.) were freshly dissolved in 0.9% NaCl; the pH was adjusted to 5.5 and then administered into the ventricle in a volume of 2  $\mu$ l. The dosage of 5HT is in terms of the free base. Controls for the 5HT-treated animals received 40  $\mu$ g of creatinine sulfate while controls to quipazine were injected with 0.9% NaCl. In all cases the intraventricular injection was given over a period of approximately 60 sec.

In two experiments the animals were injected with serotonin receptor blocker, methysergide maleate (gift of the Sandoz Laboratories), 10 mg/kg ip, 60 min before the intraventricular administration of either 5HT or quipazine.

After centrifugation of the heparinized blood samples, plasma was collected and stored frozen at  $-20^\circ$  until assay. Concentration of GH in the samples was determined by the NIAMDD radioimmunoassay system for rat GH.<sup>3</sup> All samples were measured in duplicates at two different dilutions. The results are expressed in nanograms per milliliter in terms of the RP-1 GH standard provided with the kit.

The statistical significance of the results was evaluated by the paired *t* test for sequential changes within the same group and by Student's *t* test for differences between two groups for a particular time.

**Results and Discussion.** Intraventricular injection of 5HT, 4 or 20  $\mu$ g, caused a significant

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n of plasma GH levels, which was t at 10 min and persisted throughout tion of the experiment (Table I). The onse was related to the dose of 5HT he peak levels at 30 min were signif- higher in animals receiving 20  $\mu$ g of n in the animals injected with 4  $\mu$ g. 1 of creatinine sulfate to control ani- l not influence plasma GH. Pretreat- the rats with methysergide had no e on the preinjection GH levels, but letely abolished the GH-stimulating intraventricular 5HT.

izine also induced elevation of GH. However, in comparison with ct of 5HT, the secretory responses layed and a dose-related increase ap- only at 30 min after administration of g which persisted until the 60-min t of the experiment (Table II). The ory effect of quipazine was abolished eatment of the animals with methy-

Intraventricular administration of iCl in the group of controls had no 1 plasma GH.

hink that our results provide strong

evidence that activation of the central sero- toninergic system promotes secretion of GH in the rat. This conclusion is most directly supported by the GH-stimulating effect of intraventricular administration of 5HT. Sup- pression of the effect of 5HT with serotonin receptor blocker, methysergide, lends addi- tional support to this conclusion.

There is considerable evidence that quipa- zine activates the central serotonergic sys- tem (10-12) probably by a combination of several effects, which include activation of serotonin receptors, inhibition of serotonin reuptake by serotonergic nerve terminals, and possibly enhanced release of serotonin (7, 13-15). It is, therefore, highly probable that the stimulation of GH secretion follow- ing intraventricular administration of quipa- zine originated in the activation of the central serotonergic system. The similarity between the effect of 5HT and quipazine as well as the fact that the effect of both drugs was suppressed by methysergide also speaks for this conclusion.

Difficult to explain is our observation that the GH-stimulating effect of quipazine was

E I. THIRD VENTRICULAR INJECTION OF SEROTONIN OR SYSTEMIC ADMINISTRATION OF METHYSERGIDE BY INTRAVENTRICULAR SEROTONIN ON PLASMA GH LEVELS (NANOGRAMS PER MILLILITER OF PLASMA).

Treatment and dose	Preinjection	Time after injection (min)		
		10	30	60
creatinine sulfate, 40 $\mu$ g (7) <sup>a</sup>	33.2 $\pm$ 1.5	28.9 $\pm$ 4.0	30.8 $\pm$ 2.8	34.0 $\pm$ 2.0
, 4 $\mu$ g (4)	31.3 $\pm$ 3.2	51.4 $\pm$ 7.6*	54.6 $\pm$ 6.0*	55.6 $\pm$ 12.0*
, 20 $\mu$ g (8)	27.8 $\pm$ 4.4	54.7 $\pm$ 9.0*	103.6 $\pm$ 6.5**	55.6 $\pm$ 3.4*
gide, <sup>b</sup> 10 mg/kg, + serotonin, 5)	24.5 $\pm$ 4.6	17.5 $\pm$ 6.3	25.8 $\pm$ 3.2	38.7 $\pm$ 7.5

ber of rats per group.

ysergide was given ip in a volume of 0.1 ml of saline 1 hr before third ventricular injection.

.05 vs preinjection level.

.0001 vs preinjection level.

E II. THIRD VENTRICULAR INJECTION OF QUIPAZINE OR SYSTEMIC ADMINISTRATION OF METHYSERGIDE FOLLOWED BY INTRAVENTRICULAR QUIPAZINE ON PLASMA GH LEVELS (NANOGRAMS PER MILLILITER).

ment and dose	Preinjection	Time After injection (min)		
		10	30	60
, <sup>a</sup> 2 $\mu$ l	27.0 $\pm$ 3.3	30.6 $\pm$ 1.1	29.8 $\pm$ 3.2	30.3 $\pm$ 0.8
, 4 $\mu$ g (5)	31.0 $\pm$ 1.5	27.2 $\pm$ 2.1	62.6 $\pm$ 8.9*	67.2 $\pm$ 4.1*
, 20 $\mu$ g (4)	33.5 $\pm$ 2.3	26.3 $\pm$ 2.3	105.4 $\pm$ 2.6**	80.6 $\pm$ 9.2**
gide, 10 mg/kg, + ne, 20 $\mu$ g (4)	30.6 $\pm$ 2.1	23.6 $\pm$ 1.2	26.0 $\pm$ 0.6	27.0 $\pm$ 0.6

Table I.

ysergide was given as in Table I.

.05 vs preinjection level.

.0001 vs preinjection level.

delayed as compared with the effect of 5HT. This delay is probably not caused by different pharmacodynamic properties of quipazine, because both drugs induce activation of prolactin secretion, attaining peak levels 10 min after intraventricular administration (unpublished results). It is, therefore, possible to speculate that quipazine, in addition to activation of the central serotonergic system, may have a short-lasting effect of another kind which is inhibitory to GH secretion.

Our results obtained with the intraventricular administration of 5HT in unanesthetized free-moving animals confirm the earlier work of Collu *et al.* (6) on animals anesthetized with urethane. To our knowledge this is the first report on the GH-releasing effect of quipazine.

**Summary.** Intraventricular injection of 5HT (4 and 20  $\mu$ g) in unanesthetized, unrestrained male rats fitted with permanent intrajugular cannulas for withdrawal of blood samples caused a dose-related elevation of plasma GH levels. Similar effects were also observed following intraventricular injection of the serotonin receptor agonist, quipazine. The GH-releasing effect of both drugs was abolished by a serotonin receptor blocker, methysergide. It is concluded that activation of the central serotonergic system stimulates GH secretion in the rat.

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## Effects of Ethanol on the Absorption and Retention of Lead (40317)

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requent clinical association of plumb-  
increased alcohol intake has sug-  
that ethanol may augment lead ab-  
and toxicity. This investigation was  
ten to determine the effects of acute  
chronic ethanol administration on lead  
on and excretion.

*Materials and methods.* Male albino rats of  
gen-free Wistar strain weighing 200  
g at the time of absorption measure-  
intravenous lead injection were used  
periments. The principles of labora-  
tional care as promulgated by the Na-  
tional Research Council were observed. All  
were housed in polypropylene cages  
on absorbent bedding in a room pro-  
vided with automatically controlled temper-  
ature and lighting. The rats were given a  
pelleted laboratory chow (Wayne  
Lab-Blox, Allied Mills, Inc.) fed *ad libitum*.  
Filtered deionized water was supplied  
to all animals except in some experiments in  
which 10% ethanol (v/v) was substituted for

water. Absorption studies were performed  
by measurement of total body radioactivity  
in all animal whole-body liquid scintil-  
lation detector (Packard-ARMAC). The ra-  
tios utilized were obtained from New  
England Nuclear as  $^{203}\text{Pb}$  acetate (sp act  
100 Ci/mg of lead) or  $^{210}\text{Pb}$  nitrate (sp  
act 100 Ci/mg of lead). Because of its half-  
life of 22 years,  $^{210}\text{Pb}$  was selected for use in  
long-term studies only;  $^{203}\text{Pb}$ , having a half-  
life of 42 days, was used for all other exper-  
iments. All measurements of radioactivity  
were corrected for radiodecay by comparison  
with an appropriate standard after subtraction  
of background radioactivity. Lead absorption  
studies were performed in rats fasted  
overnight from food but not fluids. Under ip  
thoracic anesthesia (4 mg/100 g), the  
animal was tied with a silk suture to prevent  
loss of absorbed lead. A laparotomy  
was performed, the small intestine was iso-

lated proximally and distally with umbilical  
tape, and the bile duct was ligated with silk  
suture. One milliliter of radiolabeled lead-  
containing test solution was injected into the  
isolated intestinal segment. Injections were  
accomplished by entering the gut lumen  
proximal to the proximal ligature with a 21-  
gauge hypodermic needle, passing it intralu-  
minally through the ligature loop, tightening  
the ligature, and then injecting the test dose  
into the isolated segment with subsequent  
withdrawal of the needle and tying of the  
ligature. In one experiment, animals were  
administered test doses through an oroeso-  
phageal tube following laparotomy. The ab-  
domen was then closed with stainless-steel  
clips and the rats were placed in 1-quart  
vented cardboard ice cream containers. Total  
body radioactivity was measured in a whole-  
body detector and compared to a 250-ml  
water-filled plastic bottle containing a test  
dose equal to that injected into the animals.  
Four hours after administration of the test  
dose, each animal was killed by cervical dis-  
location. Isolated intestinal segments were  
excised from the carcass and whole-body ra-  
dioactivity was again quantified and com-  
pared to the original whole-body radioactiv-  
ity.

To assess the effects of chronic ethanol  
ingestion on lead absorption, a group of eight  
animals was given 10% ethanol (v/v) as their  
exclusive source of fluids for 3 weeks while  
eight controls received water. The rats took  
food and fluids readily. While animal weights  
in experimental and control groups were ini-  
tially the same ( $140 \pm 5$  and  $141 \pm 7$  g,  
respectively), weight gain in ethanol-treated  
animals was less than that of controls ( $62 \pm$   
 $5$  vs  $84 \pm 15$  g,  $p < 0.05$ ); isocaloric pair  
feeding also results in a similar diminution in  
weight gain in animals receiving ethanol (1).  
Animals were given a test dose of  $1 \mu\text{g}$  of Pb  
and absorption was determined. Specimens  
of duodenum and liver were taken from ad-



ditional similarly prepared experimental and control animals for light and electron microscopic studies. The influence of acute alcohol ingestion was studied by the quantification of lead absorption in groups of animals receiving 1 ml of the following solutions in 50% ethanol: (1) 1  $\mu\text{g}$  of Pb; (2) 10  $\mu\text{g}$  of Pb; (3) 100  $\mu\text{g}$  of Pb; and (4) 1 mg of Pb. Controls received the same quantities of lead in aqueous solutions. Segments of duodenum from similarly prepared experimental and control rats were examined by light and electron microscopy.

To determine whether the diminished lead absorption from ethanol solutions was due to a direct effect on the intestine, 16 rats were given 1  $\mu\text{g}$  of Pb in the isolated intestinal segment. Half the animals simultaneously received 1 ml of 50% ethanol above the pyloric ligature by oroesophageal intubation. Control animals received 1 ml of saline. In an additional experiment, intestinal loops with open distal ends were injected with 50% ethanol followed after 15 min by lavage with 0.5 ml of air and 1 ml of saline and subsequent tying of the cecal ligature. Intestinal loops in controls were pretreated with saline followed by similar washing. Lead absorption experiments were then performed using 1  $\mu\text{g}$  of Pb. In a final study to assess the role of the site of absorption in lead absorption, six groups of eight animals were subjected to laparotomy with bile duct and cecal ligation. The rats in each group received 1  $\mu\text{g}$  in water or 50% ethanol (pH 4) by the following means: (1) aqueous and (2) alcoholic lead via oroesophageal tube, the solution confined to the stomach by a pyloric ligature; (3) aqueous and (4) alcoholic lead via oroesophageal tube without pyloric ligation; (5) aqueous and (6) alcoholic lead in isolated gut loop. Lead absorption was then quantified as previously described.

To study the effects of aqueous and alcoholic solutions on lead solubility, 100 ml of each of the following solutions were prepared as controls: (1) 1  $\mu\text{g}$  of Pb/ml; (2) 10  $\mu\text{g}$  of Pb/ml; (3) 100  $\mu\text{g}$  of Pb/ml; and (4) 1 mg of Pb/ml. Similar solutions of lead in 50% ethanol were also prepared. One microCurie of  $^{203}\text{Pb}$  was added to each 100-ml solution and the pH was adjusted to 2.0. After mixing, 1 ml of each of the resulting solutions was

removed as a standard. Each solution was titrated against 0.1 *N* NaOH to pH 12, 2-ml samples being removed at each pH value. Similar samples were taken returning to pH 2 with 0.1 *N* HCl. Samples taken from each titration were centrifuged at 3000 rpm  $\times$  30 min and 1 ml of supernatant was removed from each for quantification of radioactivity in a Packard auto-gamma counter. Solubility of aqueous and alcoholic lead was expressed as percentage of total lead as a function of pH. Supernatant from 50% ethanol solutions varying from aqueous to 5% were applied to Dowex G-25 columns equilibrated with 50% ethanol at the appropriate pH. Columns were counted successively to determine peaks of radioactivity which would indicate the presence of soluble lead-containing molecules.

In lead excretion experiments, experimental animals were given 10% ethanol for 3 weeks prior to injection and received water. Ethanol was continued throughout the experimental group throughout the measurements. While similar weight measurements were obtained in experimental and control rats at the time of fluid conditioning ( $143 \pm 7$  g,  $141 \pm 7$  g), again noted, ethanol-treated animals were significantly less at the time of injection ( $205 \pm 10$  g,  $196 \pm 9$  g,  $p < 0.05$ ). Each animal was anesthetized with sodium pentobarbital anesthesia (4 g) to facilitate injection of 1.0  $\mu\text{Ci}$  of  $^{203}\text{Pb}$  in 0.5 ml of 0.9% NaCl (pH 7.4) into the vein of the penis. Whole-body counts were obtained immediately after dosing and at intervals thereafter. Body retention of  $^{203}\text{Pb}$  was calculated by comparison of body counts with correction for decay by comparison to a standard. At the termination of excretion studies 4 weeks after dosing, whole-body radioactivity was measured for electron microscopic studies. Animals were fixed in 2% glutaraldehyde, postfix fixed in osmic acid, and embedded in Araldite. Sections 150 to 200  $\text{\AA}$  thick were stained with saturated uranyl acetate and lead citrate and examined using an RCA EMU4 electron microscope. Thick sections (1  $\mu\text{m}$ ) were stained with toluidine blue. Additional special sections for light microscopy were fixed in 10% formalin, paraffin embedded, and stained with toluidine blue and eosin.

absorption and excretion experiments of eight animals were used. Except as above for animals receiving ethanol orally, there were no differences in animal weights among the various compared in this study. For absorptions, all rats received 1 ml of a lead solution adjusted to pH 4.0, which is the pH of gastric contents and increases lead solubility. All lead quantities were expressed as grams of elemental lead as 1  $\mu$ Ci of  $^{203}\text{Pb}$  or  $^{210}\text{Pb}$  was used as a radioactive label for each rat. Data are expressed as means and standard errors of the mean. Statistical comparisons were made by Student's two-tailed *t* test for unpaired

tests. Chronic ethanol ingestion significantly reduced the absorption of a single dose of aqueous lead. While animals receiving water as fluid source for 3 weeks absorbed 1.6% of a test dose of 1  $\mu$ g of Pb, those fed with 10% ethanol for 3 weeks absorbed only  $15.2 \pm 2.9\%$  ( $p < 0.05$ ). While from animals receiving alcohol for 3 weeks showed moderate fatty change, no light microscopic or ultrastructural changes were noted in the duodenal mucosa of the same animals illustrated in Fig. 1, the absorption from aqueous solutions was significantly greater than that from ethanol solutions at concentrations of 1 and 10  $\mu$ g of Pb ( $p < 0.005$ ,  $p < 0.005$ ). At lead concentrations of 100 and 1 mg of Pb/ml, absorption from alcoholic solutions appeared to be greater than from controls but the differences were not significant ( $p = 0.20$ ,  $p = 0.10$ ). Duodenal mucosa from animals receiving 50% ethanol acutely with or without showed disruption of villous tips, pyknotic nuclei, and increased villous invasion by mononuclear cells. By electron microscopical examination of microvilli, mitochondrial cristae, and irregularity of mitochondrial membranes were noted in addition. No abnormalities were noted in animals given saline or aqueous solutions. The solubility of  $^{203}\text{Pb}$  in aqueous solutions is shown in the upper half of Table 2. Lead is more soluble in acid solutions and increasing amounts are precipitated at increasing pH. As illustrated in the lower half of Table 2, little change in radiolead solubility occurs when 50% ethanol is used as a

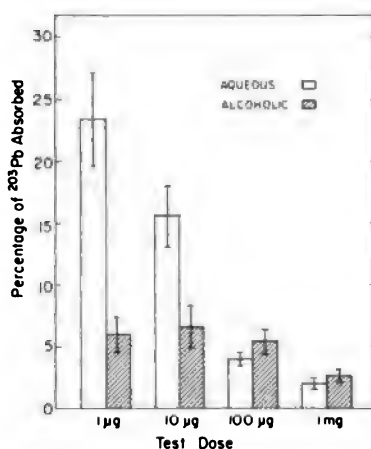


FIG. 1. The acute effects of ethanol administration on the absorption of a single dose of lead chloride.

carrier. Application of supernatants obtained in these experiments to Sephadex G-25 columns revealed no evidence of lead-containing macromolecules.

Since animals receiving ethanol on both an acute and chronic basis appeared to have diminished lead absorption unattributable to reduced lead solubility or macromolecule formation in the presence of alcohol, additional experiments were performed to determine whether at least part of this inhibitory effect was due to a direct effect of ethanol on intestinal mucosa. Rats with a pyloric ligature simultaneously administered 1  $\mu$ g of Pb in the intestinal loop and 50% ethanol in the stomach showed lead absorption (Fig. 3) which did not significantly vary from that observed in control animals. Absorption of lead in animals with ethanol-pretreated intestinal loops, however, was significantly less than that seen in rats with saline-pretreated gut loops ( $3.9 \pm 0.5$  vs  $13.5 \pm 1.5\%$  control,  $p < 0.0005$ ). As shown in Table I, only small quantities of aqueous or alcoholic lead were absorbed by the stomach ( $2.5 \pm 0.7$  and  $2.1 \pm 0.4\%$ , respectively). The absorption of lead in aqueous solution by the intestine ( $30.6 \pm 1.5\%$ ) was significantly higher than that of lead in alcoholic solution ( $8.2 \pm 0.8\%$ ,  $p < 0.005$ ) and is similar to findings shown in Fig. 1. When alcoholic lead solutions were given via oroesophageal intubation such that both stomach and intestine could act as absorptive sites, lead absorption increased to  $22.4 \pm 3.2\%$ . This value was less, however, than lead

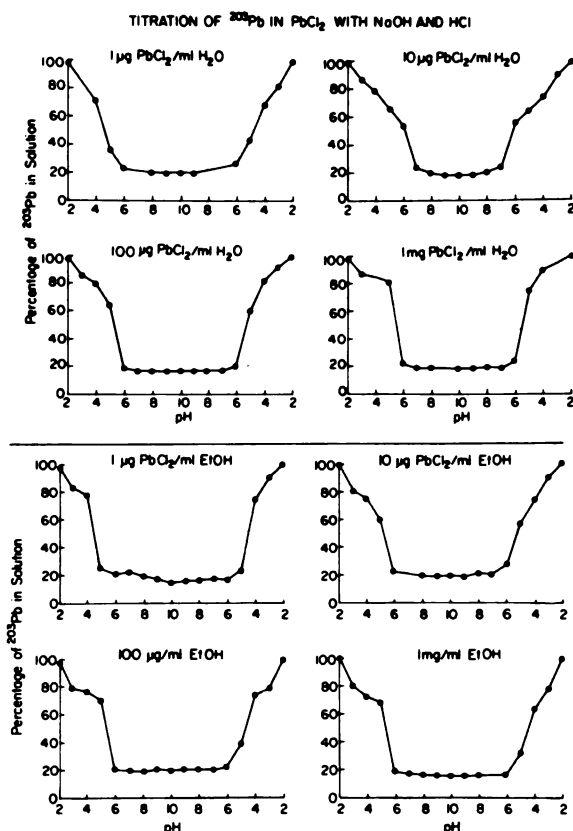


FIG. 2. The solubility of  $^{203}\text{Pb}$  in  $\text{PbCl}_2$  as affected by pH. Upper diagrams indicate solubility in aqueous solutions; lower diagrams indicate solubility in 50% ethanol.

uptake from aqueous solutions ( $28.6 \pm 1.7\%$   $p < 0.05$ ). The findings suggest that a gastric factor, perhaps ethanol-stimulated gastric acid, may act to modify lead absorption although in these experiments alcoholic lead uptake remained significantly less than controls.

As seen in Fig. 4, the excretion of lead in animals chronically receiving 10% ethanol did not significantly vary from control animals at any time during the experiment. Both groups showed an initial rapid phase of lead elimination during the first week after injection, in which time about one-half of the administered dose was excreted. This was followed by a slower phase of lead loss from the body. By using a best fit slope derived from mean-square analysis plotted on semi-logarithmic graph, the half excretion time for lead remaining after Day 7 was approximately the same for each group, about 160 days.

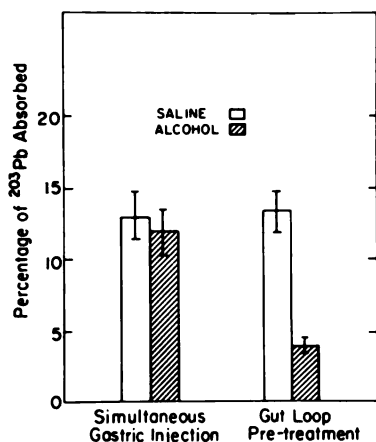
**Discussion.** A variety of clinical reports of lead poisoning in heavy consumers of alcohol (2-4) has suggested that ethanol may enhance lead accumulation and potentiate its toxic manifestations. While Mahaffey *et al.* (1) concluded that there was little synergism of ethanol and lead as measured by morphologic and biochemical parameters of lead toxicity, no studies to date have directly measured the effects of alcohol on lead absorption or retention. The results of this study indicate that: (1) the acute and chronic administration of ethanol inhibits the ability of the rat small intestine to absorb lead; (2) the effect does not seem attributable to diminished lead solubility in alcohol; (3) the inhibitory effect may be related, in part, to the direct toxicity of ethanol on intestinal mucosa; and (4) chronic ethanol ingestion does not appear to alter the excretion of lead given as a single intravenous dose. While the mechanism of lead absorption is unknown, Krawitt (5, 6)

added that acute or chronic ethanol administration inhibited calcium transport in rat gut sacs and that this effect was associated with direct mucosal toxicity. Since it is known that one or more intestinal mechanisms important in calcium mucosal binding and transfer may participate in lead absorption (7), a similar direct toxic effect on intestinal mucosa may be responsible for the diminished lead absorption found in these experiments. Whether the anatomic damage to intestinal mucosa observed in these experiments after acute ethanol administration is responsible for the diminished lead absorption after acute or chronic ethanol administration cannot be determined at present. The absence of obvious mucosal damage in rats chronically fed ethanol suggests that acute or chronic alcohol exposure may diminish lead absorption by different mechanisms. Workers, however, have noted ultrastructural changes in small intestinal mucosa following more prolonged low-level ethanol exposure (8). While there is evidence that a genetic factor may modify the absorption of

alcoholic lead from stomach and intestine, the lead absorption does not exceed that observed in aqueous lead control animals.

Factors enhancing the susceptibility to lead poisoning have been reviewed (9). Several dietary deficiencies common among heavy alcohol users have been established as capable of potentiating the manifestations of lead toxicity. While protein deficiency reduces lead absorption (11), it produces greater susceptibility to lead toxicity (12, 13). Dietary calcium deficiency increases lead retention (14, 15) and potentiates morphological and biochemical parameters of lead poisoning (16) but does not alter lead absorption (7). Iron deficiency both enhances lead toxicity (17) and increases lead absorption (10, 11). The effects of ascorbic acid, pyridoxine, and other micronutrients on lead metabolism and toxicity are not known with certainty (9).

Since these experiments indicate that acute or chronic ethanol exposure does not increase lead absorption, particularly at concentrations commonly seen in lead-containing "moonshine" whiskey (1–10  $\mu\text{g}$  of Pb/ml) (18), the apparent synergism of lead and ethanol reported in alcoholics may be related to increased lead exposure (lead-contaminated illicit whiskey or industrial environments) and/or nutritional deficiencies as previously concluded (1). These studies suggest that chronic ethanol ingestion does not alter



3. The intestinal absorption of a single dose of lead-210 in rats without prior ethanol exposure as determined by gastric injection of saline or 50% ethanol and by pretreatment of the intestinal loop by saline or ethanol (right).

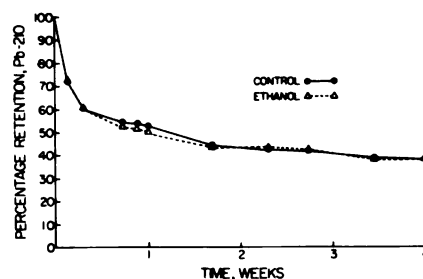


FIG. 4. The whole-body retention of lead following a single intravenous dose of lead-210 in rats chronically ingesting 10% ethanol.

TABLE I. EFFECT OF ABSORPTIVE SITE ON ABSORPTION OF LEAD FROM AQUEOUS AND ALCOHOLIC LEAD SOLUTIONS.

Solution	Site of absorption					
	Stomach		Stomach and intestine		Intestine	
	Aqueous	Alcoholic	Aqueous	Alcoholic	Aqueous	Alcoholic
Absorption (%)	2.5 $\pm$ 0.7	2.1 $\pm$ 0.4	28.6 $\pm$ 1.7	22.4 $\pm$ 3.2	30.6 $\pm$ 1.5	8.2 $\pm$ 0.8

the elimination of small quantities of lead administered as a single intravenous dose. Although there are no previously published reports of the effects of ethanol on lead excretion, the variety of renal lesions seen in plumbism and the known augmentation of lead-induced renal abnormalities by alcohol (1) suggest that diminished excretion may be of significance only when large quantities of lead are involved.

**Summary.** To determine the effects of acute and chronic ethanol ingestion on the absorption of lead, experiments were performed using an *in vivo* isolated gut loop technique. Acute administration of 50% ethanol significantly reduced the absorption of lead at concentrations of 1 and 10  $\mu\text{g}$  of Pb/ml. This effect appears to be independent of lead solubility in alcohol and is associated with structural changes in intestinal mucosa, suggesting toxicity. Absorption of a single dose of lead was also diminished in animals chronically exposed to ethanol. Elimination of a single intravenous dose of lead was not affected by chronic alcohol ingestion. These findings suggest that the clinically reported synergism of lead toxicity and ethanol is related not to increased lead absorption or diminished lead excretion but to nutritional deficiencies and increased lead exposure among some alcoholics.

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## Scatter Characteristics of Erythroid Precursor Cells Studied in Flow Analysis (40318)

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Flow analysis is a powerful new tool to study characteristics of individual cells in suspension (1). The presently available flow instruments measure either scatter of monochromatic light by cells or fluorescence of isotope-labeled cell structures. In addition, light scatter (LS) or fluorescence can be a discriminating parameter by which cell populations can be separated electronically for further study.

Bone marrow is a complex mixture of cells including all developing myeloid lines (granulocytes, erythrocytes, megakaryocytes, and lymphocytes) as well as lymphocytes. Effective study of these cell types requires samples enriched with an individual cell type. Flow instruments to sort a cell type, based on either LS or fluorescence, have been made. Fluorescence usually requires a fluorescent alteration and killing of cells. LS study is cell-sparing, sorted cells are chemically unaltered and possibly biologically active. Studies of bone marrow by flow analysis have shown the possibility of separating selected cell types of marrow (2, 3). In previous reports from this laboratory (3), emphasis has been placed on sorting granulocyte precursors of varying stages of development. In this report, the utility of separating erythrocytes and erythroid precursors in relatively pure form is discussed. This was accomplished by a preliminary isopycnic fractionation of rabbit bone marrow on density gradients followed by flow analysis of the gradient fractions, using LS to determine sorting parameters.

**Materials and methods.** *Preparation of marrow fractions.* Rabbit marrow was removed from long bones, filtered, and washed as previously described (4) except that hypotonic lysis of erythroid precursors was not performed.

*Density gradient fractionation of bone marrow.* Washed bone marrow cells were sus-

pended in Ficoll/Hypaque solution and dispersed in a linear density gradient formed with the Beckman gradient former. The mixing solutions had densities of 1.0478 and 1.1579 g/ml. Gradients were formed in 13-ml tubes for the SW41 rotor and the cells were separated isopycnically during a 40-min centrifugation at 4300g.

*Flow analysis and electronic cell sorting.* Fractions from the preliminary isopycnic separation of cells were analyzed with a Coulter Electronics Company TPS-1 sorter. Cells were analyzed at a flow rate of 1000 to 3000 cells per second and LS histograms were generated as described previously (3). The distinct and reproducible distributions in the LS histograms were used to set electronic sort windows by which 100,000 cells were sorted in each of two windows simultaneously.

Sorted cells were collected in fetal calf serum and collected on microscope slides in a Shandon cytocentrifuge. Differential cell counts were performed after staining with Wright's stain.

**Results.** *Light scatter profiles of bone marrow cells at differing buoyant densities.* Blood cell precursors of the bone marrow, both erythroid and myeloid, increase in buoyant density as they mature. Thus a preliminary separation of bone marrow cells by isopycnic sedimentation in Ficoll/Hypaque gradients allows collection of gradient fractions near the top of the gradient which are rich in immature cells and fractions of increasing maturity progressing to the bottom of the gradient (4).

The cells recovered from each density gradient fraction (I-X) were subjected to flow analysis. The LS histograms, with the cell numbers on the ordinate and increasing LS intensity on the abscissa, are shown in Fig. 1. For clarity, 4 of the 10 gradient fractions which best illustrate the typical changes in the profiles from top to bottom of the gra-

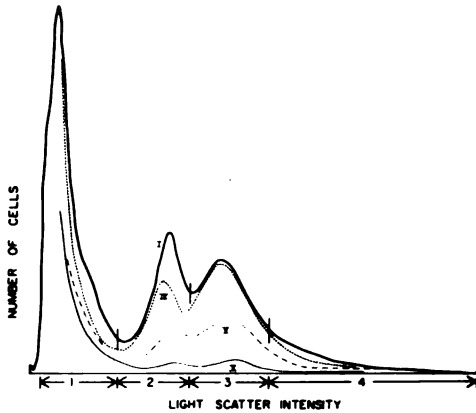


FIG. 1. Light scatter (LS) histograms of rabbit marrow cells. Cell number is plotted on the ordinate and increasing LS is indicated on the abscissa. Arabic numerals refer to limits of windows for electronic sorting of cells. Roman numerals refer to LS patterns of fractions derived from preliminary density gradient fractionation of the marrow (fraction I at top, fraction X at bottom of the density gradient).

dients are shown. The Arabic numerals on the abscissa designate sort fractions and indicate the segments under the LS profile chosen for individual electronic sorts. In gradient fraction X, too few cells were available for a fourth sort fraction.

The pattern consists of distributions representing distinct classes of cells with similar LS properties. The peak included in sort fraction 1 dominates in terms of cell number. Each curve is adjusted to show the peak at the top of the histogram so that the relative proportions of the sort fractions can be compared.

In gradient fraction I (top of the density gradient) the peak included in sort fraction 2 is more prominent than the third peak (sort fraction 3), but both peaks are similar in gradient fractions closer to the bottom of the density gradient, and both are small compared to the peak in sort fraction 1 at the bottom of the gradient.

In terms of total cells, 46% of the cells in gradient fraction I are erythroid, over half of which are nucleated. Erythrocytes make up 84% of the cells of the gradient fraction X, but only 3% of these are nucleated.

The differential counts of individual sort fractions in Fig. 2 show the distribution of cell classes in several sort fractions derived

from each density gradient fraction. The differential cell counts from each sort fraction are reported in three categories, represented by the three bars under each sort designation in Fig. 2. The "lymph" bar indicates lymphocytes and smudged nuclei (shaded portion), which may sort with lymphocytes. The "RBC" bar indicates erythrocytes and erythroid precursors. The latter are indicated by shading and "nRBC". The third bar ("gran") in each sort indicates granulocytes, mature polys, and their precursors.

Sort 1 is predominantly an erythroid fraction, sort 2 is enriched with nucleated red cells, and sorts 3 and 4 are primarily granulocyte fractions. If sort fraction 2 of gradient IV (mid-gradient) is chosen, a sample of cells is obtained which is over 90% erythroid, 64% of the cells being nucleated erythroid precursors.

An example of this fraction is shown in Fig. 3. This photomicrograph shows a group of nucleated erythrocyte precursors and one larger cell which may be lymphoid. The granulocytes chiefly responsible for the LS peak in sort fraction 3 are more mature than those which predominate in sort fraction 4. The larger less mature granulocytes scatter more light than the more mature cells (3).

**Discussion.** The scatter by cells of an incident beam of light is determined in part by the size of the cell, but also depends on reflection from cell surfaces, phase-shift in light passing around or through the cell, and diffraction of light by internal structures within the cell. The instrument involved in this study utilizes a light detector which collects light scattered  $2^\circ$  to  $20^\circ$  from the incident beam. It is known that cell size is the most important determinant at low angles of scatter ( $2^\circ$  to  $5^\circ$ ) and presumably internal structure plays a greater role in determining the intensity of scatter at larger angles (5).

It is clear from these studies that cell size is not the only determinant of scatter. The mature erythrocytes and reticulocytes scatter less light than any other cell type in the marrow, and there is a distinct separation of peaks of nucleated and non-nucleated erythrocytes. Since the size of the maturing red cell precursors decreases in a continuous fashion, if size were the major determinant of LS, there would be one broad, continuous peak

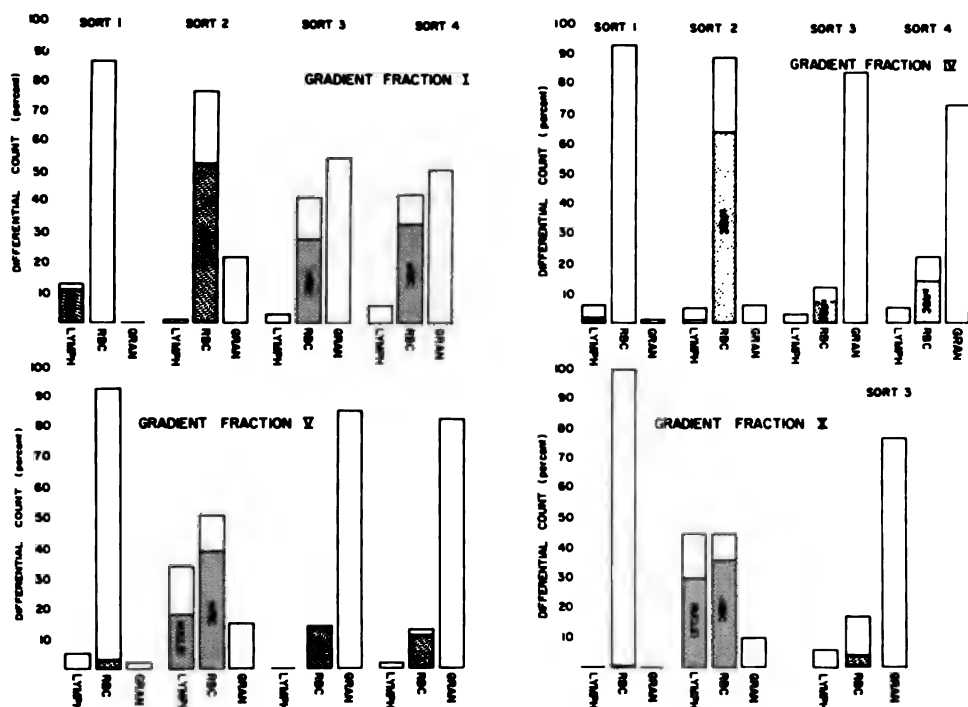


FIG. 2. Differential cell counts of cells sorted from density gradient fractions whose LS profiles are seen in Fig. 1. Each sort fraction contains cells obtained from the abscissa in Fig. 1. Each sort fraction is reported as lymph (lymphocytes and smudged nuclei), RBC (erythrocytes and nuclear RBC), and gran (neutrophils and their precursors). In gradient fraction X only three sort fractions were collected.



FIG. 3. Photomicrograph of sort fraction 2 of density gradient fraction IV. Cells shown are nucleated erythrocyte precursors and one probable lymphoid cell. Original magnification,  $\times 1000$ .



of erythroid LS. Instead, discrete distributions were observed (Fig. 1). This was also evident in other LS studies from our laboratory (3) in which it was shown that lymphocytes of various sizes were found to have very similar LS properties. It is evident that the character of the nucleus is an important LS determinant.

Granulocytic cells tend to scatter more light than erythrocytes, normoblasts, or lymphocytes. This is due no doubt to both greater cell size and much greater complexity of cytoplasmic organelles.

It is evident that LS of cells, especially when combined with separation based on buoyant density differences, is a useful means of isolating erythrocyte precursors for study.

**Summary.** Light scatter (LS) differences among cells of rabbit marrow was studied by flow analysis using a Coulter two-parameter cell sorter. A preliminary fractionation of the marrow into samples enriched with cells of varying degrees of maturation was accomplished in Ficoll/Hypaque density gradients.

Subsequent study of each of these samples in flow analysis demonstrated unimodal profiles which distinguished erythrocyte from nucleated erythroid precursor and granulocyte precursors. The combination of these procedures made it possible to obtain fractions of erythroid precursors with as high as 90% erythroid cells, two-thirds of which were nucleated precursors.

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## Salbutamol as a Topical Anti-inflammatory Drug (40319)

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The initial events in acute inflammation are the release of histamine from mast cells in response to tissue injury or antibody complexes. Histamine causes vasodilation and increased permeability of blood vessels. Local reddening and edema appear as secondary characteristics of inflammation (1). Drugs that inhibit histamine release prevent or reduce tissue inflammation. Inhibition of histamine release is accomplished partly by increasing the cellular cyclic adenosine monophosphate (2). Anti-inflammatory steroids stimulate adenyl cyclase to convert adenosine triphosphate to cAMP, and  $\beta$ -adrenergic agonists stimulate adenyl cyclase at the  $\beta$ -adrenoceptor (3).

Hydrocortisone (17 $\alpha$ -hydroxycorticosteroid) is used effectively to reduce inflammation; however, salbutamol has several distinct advantages. Salbutamol (2-(4-chlorophenyl)-2-(4-hydroxyphenyl)-1,3-dioxane-5-carboxylic acid) is a relatively specific  $\beta_2$ -agonist and selectively stimulates  $\beta_2$ -adrenergic receptors (4). The cardiovascular and central nervous system effects of other  $\beta$ -adrenergic agonists are caused in part by stimulation of the receptors which are prevalent in these tissues.

We report the local anti-inflammatory activity of salbutamol when applied topically to inflamed rat ears.

**Materials and Methods.** This method of inducing local inflammation in rat ears by croton oil is essentially that of Tonelli *et al.* (5). A 10% (v/v) croton oil solution in absolute ethanol is applied by micropipet to the outer surface of both ears (0.05 ml each). The ears become edematous in 3 to 6 hr and remain so for up to 48 hr. Inflammation is measured by cutting off the ears

at 5.5 hr and weighing them. Drugs are usually applied simultaneously in the croton oil-ethanol mixture. In some cases, as noted, drugs are applied after the croton oil. Male Sprague-Dawley rats (200-240 g) are used. Untreated control rats provide the weight of normal nonedematous ears. Croton oil-treated rats demonstrate the extent of inflammation in the absence of drugs. Hydrocortisone (1%), serving as a positive control, consistently inhibits inflammation by 80 to 100%. Data are expressed as milligrams of edema of both ears, that is, the increase in weight of both ears over the untreated controls. The weights in each group are averaged and the standard error of the mean is calculated (depicted by vertical line extensions on the graphs).

**Results.** Local inflammation is inhibited totally by hydrocortisone and salbutamol when they are applied topically to the ears at 1 to 2% (w/v) in the croton oil solution (Fig. 1). Croton oil causes the ears to gain an average of 155 mg in the absence of any anti-inflammatory agent. Drug concentrations of 0.1% reduce the edema by 80%. When drugs are applied to a distant shaven area of the back, anti-inflammatory activity still occurs but higher concentrations are required (Fig. 1B).

Hydrocortisone and salbutamol reduce local edema even when applied after the inflammation reaction is in progress (Fig. 2). In the case of salbutamol, significant reduction of inflammation is obtained when given up to 2 hr after application of the croton oil. Hydrocortisone is not as effective when given this late in the development of acute inflammation.

Salbutamol is found to be inactive orally in our model (Fig. 3). Doses of up to 35 mg/kg body wt, delivered orally by stomach tube, failed to significantly inhibit ear edema.

Propranolol (a  $\beta$ -adrenergic receptor blocking agent) interferes with the ability of salbutamol to inhibit inflammation, but di-

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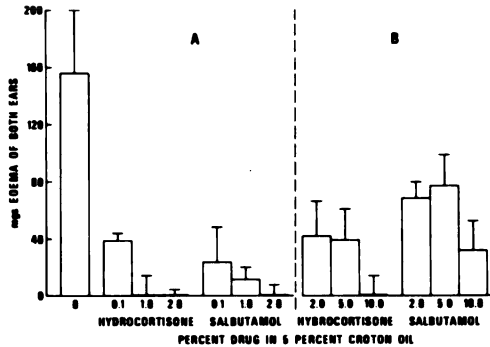


FIG. 1. Local and systemic anti-inflammatory activity of hydrocortisone and salbutamol. The drugs are applied directly to the ears (A) or to a shaven area on the back (B). In both A and B, the croton oil was applied to the ears to induce inflammation. In this and subsequent graphs the averages of five animals per group are presented, and the vertical line extensions represent the standard errors of the mean.

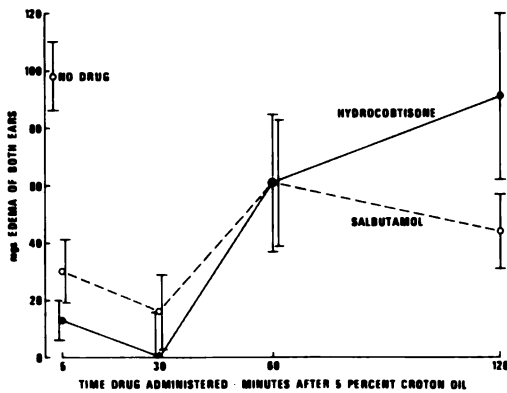


FIG. 2. The effects of salbutamol and hydrocortisone on local inflammation when they are administered during the course of the inflammation reaction. Croton oil was applied to the ears to induce inflammation, while salbutamol (1%) and hydrocortisone (1%) were also applied but at various times after the croton oil.

benamine (an  $\alpha$ -adrenergic receptor blocking agent) has no influence (Fig. 4). Neither propranolol nor dibenamine prevents the anti-inflammatory activity of hydrocortisone.

**Discussion.** The need exists for a locally active anti-inflammatory drug that can be applied directly. Salbutamol (Ventolin, Allen and Hansbury) is used in foreign countries in the management of asthma (6). Green (7) has reported that salbutamol, injected ip, could reduce inflammation both in the mouse peritoneum induced by acetic acid and in the rat hindpaw edema induced by carrageenin. He also demonstrated that the activity is not

mediated by release of adrenal corticosteroids.

Salbutamol is very effective in the prevention of local inflammation. Although it is orally active in our model of inflammation salbutamol is effective if applied directly to the inflamed site or at a remote site. This suggests that the drug is readily absorbed in the circulatory system; however, larger concentrations are required if the drug is applied at the site of inflammation.

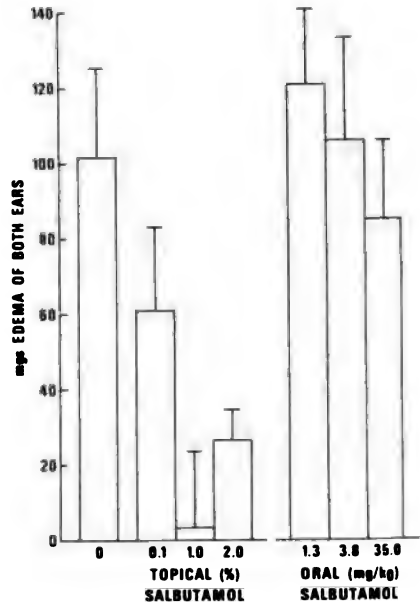


FIG. 3. Topical and oral activity of salbutamol on local inflammation. Salbutamol was applied directly to the ears, or given orally by stomach tube, at various doses, 30 min prior to the croton oil.

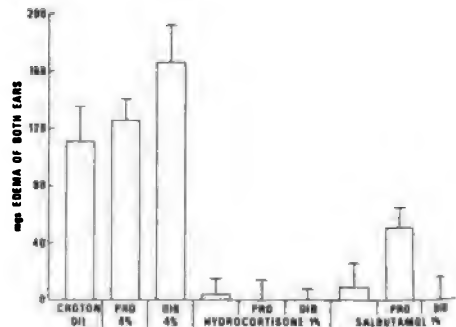


FIG. 4. Effects of 4% propranolol (pro) and 4% benamine (dib) on the local anti-inflammatory activity of hydrocortisone and salbutamol. Croton oil, propranolol, and dibenamine were given independently (controls) and in combination with the drugs (simultaneous application, including croton oil).

## SALBUTAMOL IS ACTIVE IN INFLAMMATION

ion of salbutamol as a bronchodilator treatment of asthma is mediated by  $\beta_2$ -adrenergic receptors in the bronchus and  $\beta_1$ -adrenoceptors in the mast cells involved in the action of salbutamol. The  $\beta$ -blocking agent propranolol interferes with the ability of salbutamol to inhibit inflammation. Dibenamine, a  $\beta$ -blocking agent, has no effect. Hydrocortisone acts in a different manner since neither salbutamol nor dibenamine block the effect of hydrocortisone.

Salbutamol has minimal cardiac side effects are expected, because salbutamol is selective for  $\beta_2$ -receptors and has little effect on  $\beta_1$  receptors which predominate in the heart. The minimal adverse effects of salbutamol compared with other  $\beta$ -agonists for the control of asthma are discussed by Dockhorn (8) and by Dochorn (9). We have found the anti-inflammatory activity of other agents. Salbutamol was pursued because it was the most effective and because of its safety." Morrison and Farebrother (10) reported a case of salbutamol overdose but the physiological and cardiovascular effects that occur. Further studies are required but it appears safe to attempt to treat inflammatory conditions of the respiratory tract with salbutamol.

**Summary.** Using croton oil-induced edema, hydrocortisone and salbutamol have been shown to have anti-inflammatory activity when applied topically. Both drugs act to some extent when applied after the inflammation reaction is in progress. Both drugs are also active when applied to a shaven area of the back, remote from the ear inflammation. Salbutamol acts by a different mechanism than the inflammatory steroids. The advantages of salbutamol are discussed and it appears to be a useful adjunct in the treatment of inflammatory dermatoses.

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# Interaction of Ethanol and Thyroxine on Hepatic Oxygen Consumption<sup>1</sup> (4032)

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Chronic feeding of ethanol to rats has been shown to stimulate respiration by liver slices through an increase in conversion of ATP to ADP by the (Na + K)-ATPase system (1, 2). The calorogenic effect of thyroid hormones also involves stimulation of (Na + K)-ATPase (3). However, some studies have suggested that the availability of mitochondrial substrate and not ADP may determine the rate of respiration and that thyroxine (T<sub>4</sub>) enhances the availability of the substrate for mitochondrial oxidation (4).

The present study was done to investigate interrelationship between the effects of chronic ethanol ingestion and T<sub>4</sub> treatment on O<sub>2</sub> consumption by rat liver slices and isolated mitochondria. Further, the influence of the available oxidizable substrate for the ethanol and T<sub>4</sub> effects on respiration of rat liver slices was studied.

**Materials and Methods.** Thirty-two Sprague-Dawley male rats weighing 150 to 200 g were divided equally into four groups at random. Group A received tap water and group B received 20% (v/v) ethanol as the only drinking solution *ad libitum*. Group C was rendered thyrotoxic by daily ip injection of 1-T<sub>4</sub> (150 µg/100 g body wt) for 14 days. Group D received 20% (v/v) ethanol as drinking solution and T<sub>4</sub> treatment as outlined for group C. All animals were housed in individual cages, fed regular Purina Chow *ad libitum*, and weighed at regular intervals. Animals in group D lost considerable weight (see Table I) and appeared sick, although none died. In eight relatively young rats, average weight 100 g, a 25% mortality rate was observed during 20% ethanol + T<sub>4</sub> treatment and therefore present studies involved relatively larger animals.

After 14 days the animals were fasted 18 hr and then sacrificed by decapitation. Blood was collected for the estimation of serum T<sub>4</sub> levels (5). Livers were removed immediately in ice-cold oxygenated medium containing 135 mM NaCl, 4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 5 mM Tris and 10 mM glucose, pH 7.4. Liver slices 1 mm thick were prepared and their respiration was determined in a Warburg apparatus (Oxygenation Scientific). Each Warburg flask contained approximately 60 mg of tissue in 1 ml of the oxygenated medium mentioned above. Respiration was measured for three consecutive 30-min periods. Thereafter 50 µl of 0.1 M succinate was added to the medium in the side arm to give a final concentration of 7 mM and respiration of the liver slices was estimated for three additional 10-min periods.

To determine oxygen consumption by isolated mitochondria instead of liver slices, mitochondria were isolated from the same animals according to the technique of Johnson and Lardy (6). An aliquot, 0.05 ml, of the mitochondrial suspension was placed in a Warburg flask containing 3 ml of incubation medium which contained 62.5 mM sucrose, 185.5 mM mannitol, 10 mM KCl, 10 mM Tris-HCl, pH 7.4, 5 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 7 mM succinate, and 83.3 µM ADP. Respiration was measured for three consecutive 10-min periods.

Respiration estimations for liver slices and mitochondria were done in triplicate for each animal. The protein content of the liver and of each mitochondrial suspension was determined by the Lowry method (7). All data were expressed as microliters of O<sub>2</sub> consumed per minute per milligram of protein and statistically analyzed by Student's *t*-test.

**Results.** Table I shows mean ± SEM of body weight and serum thyroxine levels in rats receiving ethanol, thyroxine, or a combination of these two substances (*n* = 8 in each group of animals). Rats that re-

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TABLE I. EFFECT OF ETHANOL INGESTION ON BODY WEIGHTS AND SERUM T<sub>4</sub> LEVELS OF NORMAL AND THYROXINE-TREATED RATS.

Treatment	Initial body weight (g)	Final body weight (g)	Difference	Serum thyroxine (μg%)
A. Saline	166 ± 8	238 ± 8	72 ± 6	4.6 ± 0.4
B. Ethanol	179 ± 8	194 ± 9	15 ± 4*	3.9 ± 0.3
C. T <sub>4</sub>	176 ± 10	229 ± 12	53 ± 8	16.6 ± 2.4*
D. Ethanol + T <sub>4</sub>	179 ± 7	132 ± 7	-47 ± 4*	14.1 ± 2.9*

\*  $p < 0.001$  compared to controls. (saline).

either ethanol or T<sub>4</sub> gained significantly less weight than controls ( $p < 0.05$ ). Simultaneous treatment with ethanol and thyroxine produced a marked loss in body weight as compared to controls ( $176.2 \pm 7$  vs  $127 \pm 7$ ;  $p < 0.01$ ). Serum T<sub>4</sub> levels were significantly higher ( $p < 0.01$ ) in T<sub>4</sub>-treated animals and ethanol ingestion exerted no discernible effect on serum T<sub>4</sub> values.

*Effect of ethanol on O<sub>2</sub> consumption by liver slices of euthyroid and thyrotoxic rats.* Figure 1 shows that chronic ethanol ingestion decreased the rate of oxygen utilization from a control value of  $0.098 \pm 0.004$  to  $0.082 \pm 0.004$  μl of O<sub>2</sub>/min/mg of protein in liver slices of euthyroid rats. The results were significant at the 2% level. Addition of succinate to liver slices produced a marked increase in O<sub>2</sub> consumption to  $0.183 \pm 0.01$  and  $0.243 \pm 0.01$  μl of O<sub>2</sub>/min/mg of protein in controls and ethanol-treated animals, respectively. Furthermore, with succinate as oxidizable substrate, ethanol pretreatment produced an increase ( $p < 0.001$ ) in the rate of respiration instead of a depression of respiration observed with glucose as the substrate.

Figure 2 shows that in thyrotoxic rat liver slices the O<sub>2</sub> consumption was 50% greater than in euthyroid rat liver slices ( $p < 0.001$ ). Chronic ethanol ingestion decreased O<sub>2</sub> consumption by nearly 50% from  $0.147 \pm 0.004$  to  $0.071 \pm 0.005$  μl of O<sub>2</sub>/min/mg of protein ( $p < 0.001$ ) with glucose as substrate. Addition of succinate increased respiration of T<sub>4</sub>-treated rat liver slices and chronic ethanol ingestion enhanced the rate of respiration further from  $0.282 \pm 0.015$  to  $0.367 \pm 0.028$  μl of O<sub>2</sub>/min/mg of protein ( $p < 0.02$ ).

*Effect of ethanol and thyroxine on O<sub>2</sub> consumption of isolated mitochondria of rat liver.* As shown in Fig. 3, mitochondria isolated from euthyroid rat liver showed no significant difference in respiration after chronic ethanol treatment as compared to control values.

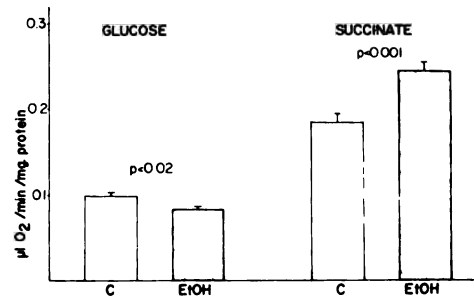


FIG. 1. Mean  $\pm$  SEM ( $n = 8$ ) oxygen consumption rate by liver slices of rats fed 20% ethanol as drinking solution or tap water (controls) for 14 days. The rate of O<sub>2</sub> consumption was estimated with liver slices in media containing glucose before and after the addition of succinate.

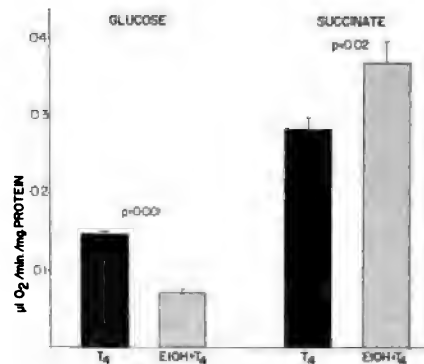


FIG. 2. Mean  $\pm$  SEM ( $n = 8$ ) oxygen consumption by liver slices of T<sub>4</sub>-treated rats that received 20% ethanol or tap water *ad libitum* for 14 days. Oxygen estimation was done as described under Fig. 1 and T<sub>4</sub> injections were given as described in the text.

However, in mitochondria isolated from thyrotoxic rat livers it was observed that chronic ethanol treatment enhanced O<sub>2</sub> consumption significantly from  $1.27 \pm 0.032$  to  $1.57 \pm 0.118$  μl of O<sub>2</sub>/min/mg of protein ( $p < 0.05$ ).

*Discussion.* Previously it has been shown that daily ingestion of ethanol (35% calorie-wise) for 21 to 27 days enhanced oxygen consumption by rat liver slices. The under-

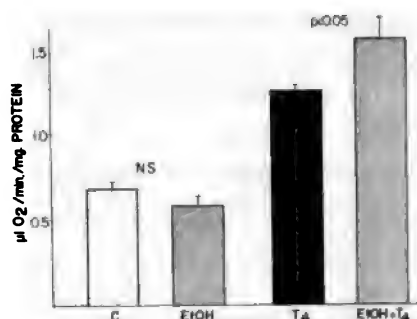


FIG. 3. Mean  $\pm$  SEM ( $n = 8$ ) oxygen consumption by mitochondria isolated from livers of euthyroid and  $T_4$ -treated rats. Both groups of euthyroid or  $T_4$ -treated rats were given 20% ethanol or tap water *ad libitum* for 14 days. Injections of  $T_4$  were given as described in the text.

lying mechanism was reported to be an increased activity of the (Na + K)ATPase activity (1, 2). The present data derived from rats consuming relatively less ethanol (i.e., 20% (v/v) as drinking solution *ad libitum* for 14 days) show that the ethanol effect on respiration of rat liver slices is dependent on the available oxidizable substrate. The  $O_2$  consumption by liver slices was increased in medium containing succinate as substrate but decreased when glucose was used instead of succinate.

Substrates can provide electrons to the respiratory chain at the beginning (the level of NADH dehydrogenase), at the middle (ubiquinone level), and at the terminus (cytochrome *c* level). Succinate which is flavin-linked provides electrons at the cytochrome *b*-ubiquinone segment and therefore bypasses energy coupling site I at the level of NADH dehydrogenase. The utilization of electrons from glucose is partly NAD-linked and thus involves energy coupling site I. The present results might be explained by an inhibitory effect of ethanol on coupling site I or on some steps prior to it. In fact, Cederbaum *et al.* (8) have shown that chronic ethanol ingestion (36% caloriewise) depresses mitochondrial respiration by damaging coupling site I.

Whereas ethanol enhanced  $O_2$  consumption in rat liver slices incubated with succinate, it did not exhibit a similar effect when isolated mitochondria from the same livers were studied. Other studies have reported a

depression of mitochondrial respiration by chronic ethanol ingestion and ascribed the effect to a damage to the respiratory chain. Furthermore, structural changes in mitochondria including swelling, disorganization of cristae, and intramitochondrial crystalline inclusion are observed after chronic ethanol treatment (9). Correlation of fat infiltration of hepatocytes has been shown (10). In the present study relatively less ethanol, and any morphological changes in mitochondria, although not documented, were perhaps insufficient to depress mitochondrial respiration. In fact, the  $O_2$  consumption by mitochondria isolated from thyrotoxic rats was enhanced by chronic ethanol treatment. Therefore, it is unlikely that the respiratory chain was damaged by ethanol as observed in rats in this study.

The calorogenic effect of thyroid hormone on liver is ascribed to an increase in energy production due to stimulation of (Na + K)ATPase activity (3). However, Prior and Buchanan (4) showed  $O_2$  consumption by liver slices was greater with succinate than with glucose and suggested that the availability of oxidizable substrate rather than the concentration of substrate controls the rate of  $O_2$  consumption. The present data show that chronic ethanol treatment decreased  $O_2$  consumption of rat liver slices when glucose was available as substrate but a converse effect occurred when succinate was added. It seems that the inhibition of energy coupling site I discussed above, was sufficient to block succinate-dependent respiration in a glutamine-containing medium. On the other hand, the increase in succinate-supported respiration of the same liver slices might be related to the increased (Na + K)-ATPase activity.

It needs to be emphasized that rats receiving ethanol +  $T_4$  lost considerable weight and appeared sick. A decrease in food intake might have resulted in a limitation of substrate for  $O_2$  utilization. The conceivable effect of malnutrition is similar to that of ethanol should be considered in the interpretation of data derived from these rats. Nonetheless, individual rat liver slices were used as its own control in terms of comparative rates of  $O_2$  utilization during two substrates, i.e., glucose and succinate.

**Summary.** Interrelationship between

of chronic ethanol ingestion and T<sub>4</sub> on O<sub>2</sub> consumption by rat liver and isolated mitochondria was investigated. The data showed that ethanol influenced O<sub>2</sub> consumption by liver slices was dependent on the available oxidizable substrate as it was decreased when estimated in containing glucose but increased in containing succinate as oxidizable substrate. The respiration of thyrotoxic rat liver was altered by ethanol in a manner different to that observed with euthyroid rat liver slices. Whereas ethanol ingestion ended succinate-supported respiration of euthyroid and thyrotoxic rat liver slices, it produced a similar effect in isolated mitochondria of thyrotoxic rat livers but not of euthyroid livers.

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# Effect of a Phosphodiesterase Inhibitor, 3-Isobutyl 1-methylxanthine, upon the Stimulatory Effect of Human Follicle-Stimulating Hormone and Human Luteinizing Hormone upon Cyclic Adenosine 3':5'-Monophosphate Accumulation by Porcine Granulosa Cells<sup>1</sup> (40321)

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A mechanism of polypeptide hormone action on target cells is to stimulate formation of cAMP which subsequently acts as an intracellular mediator of hormone action. Intracellular cAMP levels are the result of given rate of synthesis combined with a given rate of degradation or extracellular release. The cyclic nucleotide is believed to be hydrolyzed to 5'-AMP by one or more cyclic nucleotide phosphodiesterases (1). Methylxanthines have been shown to exert inhibitory effects on the action of phosphodiesterase (2, 3). We have shown previously that LH and FSH can stimulate cAMP accumulation by porcine granulosa cells (GC) and that the amount of cAMP accumulated in response to the two gonadotropins differs according to the stage of maturation of the follicle (4). In addition, observations from previous studies (4) suggest that the phenomenon of cAMP accumulation by porcine GC in response to the stimulatory effects of the gonadotropins occurs over time. For GC from small and medium follicles the intracellular cAMP accumulated in response to FSH was not observed to decline significantly in incubations of 30 min or less. The decline occurred between 30- and 60-min periods of incubation and it was during this time interval that the increase in cAMP accumulation in the incubation medium was observed to occur. For GC from large follicles the intracellular cAMP accumulated in response to LH was not observed to decline with 30- nor with 60-min incubations; however, a significant increase in the cAMP accumulation in the incubation medium oc-

curred between 30- and 60-min periods of incubation. The present studies were designed to investigate the influence that phosphodiesterase may exert on the cAMP accumulation phenomenon previously observed in porcine GC in response to the stimulatory effects of FSH and LH. In the present studies the phosphodiesterase influence was examined indirectly using a potent phosphodiesterase inhibitor.

The effects of phosphodiesterase inhibition upon cAMP accumulation by porcine GC previously have not been adequately examined. The influence of methylxanthine upon the stimulatory effects of purified hFSH and hLH on porcine GC intracellular cAMP accumulation and upon cAMP accumulation in the incubation medium was investigated. These studies enabled the determination of the relative approximate contribution of synthesis, degradation, and extracellular release to cAMP levels occurring in porcine GC during various stages of follicular maturation in response to hFSH and hLH.

**Materials and methods.** *Granulosa cell harvest.* Porcine ovaries were obtained from a local meat packing plant within 15 to 20 min of sacrifice of the animals. Granulosa cells were harvested from small (1-2 mm), medium (3-5 mm), and large (6-12 mm) follicles according to the method of Channing and Ledwitz-Rigby (5). Using dye exclusion as an indication of cell viability, the cells were counted in a hemocytometer in 0.06% trypan blue.

**Hormones and chemicals.** Highly purified hLH, LER-1705, having a potency of 3800 IU/mg and an FSH activity of 3 IU/mg, and hFSH, LER-1577<sup>c</sup>, having an FSH potency of 880 IU/mg were used. These two hormone preparations were provided by Dr. L. E. Reichert, Jr. The FSH preparation as sup-

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Dr. Reichert had been pretreated with trypsin to inactivate the contaminating LH selectively (6). The residual LH was reported to be 5.7 IU/mg using the ascorbic acid depletion assay (7). According to Amir *et al.* (8), controlled chymotrypsin digestion does not destroy FSH activity determined by the Steelman-Pohley (9).

Cells were incubated in the absence or presence of the hormones in Eagle's medium containing Earle's salts (pH 7.4; Grand Island Chemical Co., Grand Island, N.Y.), 25 mM HEPES buffer (Calbiochem), 2.2 g/liter glucose (Grand Island Biological Co.), and 1% bovine serum albumin (BSA) fraction V (Chemical Company). This was designated as Eagle's medium plus 1% BSA. 3-Isobutyl-1-methylxanthine (MIX) was purchased from the Aldrich Chemical Company and was diluted in Eagle's medium plus 1% BSA. The final concentration of MIX for incubations with the cells and for protein analysis was 0.2 mM. Both [ $^3\text{H}$ ]cAMP (4 Ci/mmol) and nonlabeled cAMP were purchased from Schwartz Bio-Research,

*Granulosa cell incubations and experimental procedures.* Granulosa cells from small and large follicles were suspended in Eagle's medium plus 1% BSA and dispensed in aliquots of  $2 \times 10^7$  cells. Cells from large follicles were dispensed in aliquots of  $5 \times 10^6$  cells. Incubations were carried out in Packard DPM-35 scintillation vials containing the appropriate hormone. When 3-isobutyl-1-methylxanthine (MIX) was used it was added to the incubation medium containing the appropriate hormone prior to the addition of the cells. The final incubation volume per vial was 1.0 ml. Three to five aliquots of cells were used for each experiment. Incubations were carried out for 30 and 60 min under conditions previously described (5). The reaction was arrested by placing the vials immediately in ice. The cells were separated from the incubation medium by centrifugation. The incubation medium was decanted and the cell pellets were subjected to 50% acetone extraction and following centrifugation the clear supernatant was de-

canted and frozen for later assay of intracellular cAMP.

*Cyclic AMP assay.* Cyclic AMP accumulation was determined by a competitive protein binding assay (10) with modifications (5, 11). Using 1.25 pmol of cAMP as a standard after every 10 unknown samples, the intra-assay coefficient of variation was less than 16% and for 30 randomly selected assays the between assay coefficient of variation was less than 15%.

*Results. Effect of MIX upon intracellular cAMP accumulation.* The presence of 0.2 mM MIX in the incubation medium did not significantly alter the control levels of intracellular cAMP in GC harvested from small, medium, and large follicles following 30- or 60-min incubation periods (Table I). Addition of 1.0 and 10  $\mu\text{g}$  of hFSH resulted in an increase in intracellular cAMP accumulation in GC from small, medium, and large follicles (Table I). In the case of cells from small follicles, addition of 10  $\mu\text{g}$  of hFSH led to a greater than 13-fold increase ( $p < 0.001$ ) in intracellular cAMP levels following 30-min incubations and a greater than 22-fold increase ( $p < 0.001$ ) following a 60-min incubation period (Tables I and II). In contrast, addition of 10  $\mu\text{g}$  of hFSH to cells from large follicles led to less than a 3-fold increase above control levels after either 30- or 60-min incubation periods ( $p < 0.001$  and  $p < 0.01$ , respectively). A small nonsignificant ( $p > 0.05$ ) potentiating effect of 0.2 mM MIX upon the stimulatory effect of 1.0 and 10  $\mu\text{g}$  of hFSH upon intracellular cAMP accumulation was observed (Table I).

Addition of hLH stimulated intracellular cAMP accumulation in GC (Table I). The stimulation was greater in the case of GC harvested from large compared to medium and small follicles. Addition of 0.2 mM MIX exerted a small nonsignificant ( $p > 0.05$ ) potentiating effect upon the LH stimulation of intracellular cAMP levels in cells from all three types of follicles (Table I). If GC were incubated for 60 rather than 30 min addition of 0.2 mM MIX still had no significant effect upon hFSH and hLH stimulation of intracellular cAMP levels (Table II).

*Effect of MIX upon cAMP released into the incubation medium.* The presence of 0.2 mM MIX in the incubation medium did not sig-

TABLE I. COMPARISON OF EFFECT OF 0.2 mM 3-ISOBUTYL 1-METHYLSXANTHINE UPON hFSH AND hLH STIMULATION OF INTRACELLULAR cAMP ACCUMULATION IN PORCINE GC DURING 30-MIN INCUBATIONS.\*

Source of GC and treatment	Intracellular cAMP (pmol/5 × 10 <sup>7</sup> cells)	
	3-Isobutyl 1-methylxanthine Absent	Present
<b>Small follicle</b>		
Control	8.1 ± 0.7	8.9 ± 0.9
0.1 µg hFSH	10.4 ± 1.3	14.2 ± 2.8
1.0 µg hFSH	62.7 ± 3.7	71.0 ± 5.6
10.0 µg hFSH	91.5 ± 9.4	99.3 ± 9.3
0.01 µg hLH	8.6 ± 2.2	8.7 ± 1.3
0.1 µg hLH	9.5 ± 1.9	12.7 ± 1.9
1.0 µg hLH	12.7 ± 2.1	15.4 ± 2.1
<b>Medium Follicle</b>		
Control	8.9 ± 1.6	8.4 ± 1.2
0.1 µg hFSH	8.0 ± 0.3	9.9 ± 0.5*
1.0 µg hFSH	38.3 ± 6.1	37.6 ± 4.5
10.0 µg hFSH	51.6 ± 4.2	40.4 ± 6.9
0.01 µg hLH	13.2 ± 3.6	11.3 ± 1.0
0.1 µg hLH	23.5 ± 3.4	30.6 ± 3.2
1.0 µg hLH	30.8 ± 1.4	36.9 ± 3.5
<b>Large follicle</b>		
Control	92.9 ± 6.2	109.6 ± 8.2
0.1 µg hFSH	85.9 ± 14.8	95.0 ± 17.7
1.0 µg hFSH	184.5 ± 10.3	191.0 ± 21.8
10.0 µg hFSH	204.3 ± 9.9	231.8 ± 20.5
0.01 µg hLH	134.3 ± 10.0	149.4 ± 31.0
0.1 µg hLH	236.5 ± 12.0	272.1 ± 13.9
1.0 µg hLH	278.1 ± 12.4	314.5 ± 13.4

\* Data are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 30 min with hFSH or hLH in the absence or presence of 0.2 mM MIX and the intracellular cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent). The differences were not statistically significant (*p* > 0.05) unless indicated.

\* *p* < 0.05.

nificantly alter control levels of cAMP released into the incubation medium by GC from any size follicle during 30- or 60-min incubation periods (Tables III and IV). Addition of 10 µg of hFSH to GC from small follicles led to a 16- and 45-fold increase in incubation medium cAMP levels following 30- and 60-min incubation periods, respectively (Tables III and IV). In the case of GC from small and medium follicles, addition of 0.2 mM MIX in the presence of 1.0 (data not shown) and 10 µg of hFSH led to a significant increase in incubation medium cAMP content (Tables III and IV). In contrast, the presence of MIX did not significantly potentiate the effect of hFSH upon cAMP accumulation in the incubation medium by GC from large follicles (Tables III and IV).

The presence of MIX brought about a significant potentiation of the stimulatory effect of 1.0 µg of hLH upon cAMP released into the incubation medium by GC from small and medium follicles following 30- and 60-min incubations (Tables III and IV). In the case of GC from large follicles the potentiating effect of MIX upon hLH stimulation of cAMP accumulation in the incubation medium was not significant (*p* > 0.05) during 30- or 60-min incubations (Tables III and IV).

After a 60-min incubation period with either 10 µg of hFSH or 1.0 µg of hLH the incubation medium cAMP levels were consistently greater than the intracellular levels in the case of cells from all three follicle types (Tables II and IV).

II. COMPARISON OF EFFECT OF 0.2 mM 3-  
1-METHYLXANTHINE UPON hFSH AND hLH  
STIMULATION OF INTRACELLULAR cAMP  
ACCUMULATION IN PORCINE GC DURING 60-MIN  
INCUBATIONS.<sup>a</sup>

Source of GC and treatment	Intracellular cAMP (pmol/5 × 10 <sup>7</sup> cells)	
	3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	3.1 ±0.2	4.3 ±0.7
10.0 µg hFSH	71.0 ±4.8	75.8 ±3.7
1.0 µg hLH	10.8 ±0.8	13.6 ±3.2
Medium follicle		
Control	3.8 ±0.3	4.1 ±0.2
10.0 µg hFSH	22.0 ±3.64	28.1 ±3.7
1.0 µg hLH	11.1 ±2.0	13.0 ±3.5
Large follicle		
Control	86.8 ±6.6	61.5 ±6.3
10.0 µg hFSH	224.1 ±30.6	264.6 ±34.6
1.0 µg hLH	369.1 ±25.2	385.5 ±44.6

are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 60 min with hFSH or with hLH in the absence or presence of 0.2 mM MIX and the intracellular cAMP levels were determined. Student's *t* test was used to compare results obtained with MIX present vs MIX absent. The differences were not significant (*p* > 0.05).

Conclusion. The lack of a significant potentiating effect of MIX on intracellular cAMP accumulation by porcine GC in response to 60-min periods of incubation with hFSH or hLH could indicate that phosphorylation of cAMP by a phosphodiesterase(s) is not a major mechanism responsible for controlling the intracellular level of cyclic nucleotide. Alternatively, it is possible that this methylxanthine does not permeate the GC plasma membrane and thus does not successfully inhibit phosphodiesterase or that the concentration employed was not sufficient to inhibit GC intracellular phosphodiesterase(s). It is evident from the findings of other investigators that concentrations of MIX ranging from 0.01 to 1.0 mM have

potentiating effects on cAMP accumulation. Methylxanthine has been observed to potentiate the effect of ACTH upon cAMP levels in rat adrenal homogenates and quarters (13) and in isolated fat cells (14). Mendelson *et al.* (12) reported that the sensitivity of isolated rat testis interstitial cells to hCG stimulation was significantly enhanced with the presence of 0.1 mM MIX and in the absence of MIX, cAMP accumulation in response to hCG was reduced in magnitude by about 60%. These investigators used the sonicated incubation mixture for assay of cAMP; thus their reported findings reflect inclusion of both the intracellular and incubation medium cAMP content and the site of the potentiating effect remains obscure. Channing (15) observed

TABLE III. COMPARISON OF EFFECT OF 0.2 mM 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF cAMP ACCUMULATION IN THE INCUBATION MEDIUM BY PORCINE GC DURING 30-MIN INCUBATIONS.<sup>a</sup>

Source of GC and treatment	Incubation medium cAMP (pmol/5 × 10 <sup>7</sup> cells)	
	3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	7.0 ±0.7	6.9 ±0.8
10.0 µg hFSH	115.3 ±3.2	143.2 ±3.5***
1.0 µg hLH	6.2 ±0.7	13.4 ±1.4***
Medium follicle		
Control	6.5 ±0.6	7.1 ±0.8
10.0 µg hFSH	25.1 ±2.9	49.3 ±3.4***
1.0 µg hLH	10.8 ±1.6	19.8 ±1.4***
Large follicle		
Control	26.3 ±4.9	27.6 ±4.6
10.0 µg hFSH	110.2 ±19.5	158.0 ±26.4
1.0 µg hLH	210.9 ±62.8	269.1 ±66.3

<sup>a</sup> Data are expressed as the means ± SE of eight observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 30 min with hFSH or with hLH in the absence or presence of 0.2 mM MIX and the incubation medium cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent).

\*\*\* *p* < 0.001.

TABLE IV. COMPARISON OF EFFECT OF 0.2 mM 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF cAMP ACCUMULATION IN THE INCUBATION MEDIUM BY PORCINE GC DURING 60-MIN INCUBATIONS.<sup>a</sup>

Source of GC and treatment	Incubation medium cAMP (pmol/5 × 10 <sup>7</sup> cells)	
	3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	4.4	6.6
	±0.4	±1.8
10.0 µg hFSH	198.6	297.7
	±12.2	±19.5**
1.0 µg hLH	28.2	68.3
	±2.3	±3.4***
Medium follicle		
Control	11.7	13.0
	±3.8	±4.8
10.0 µg hFSH	101.1	146.0
	±16.6	±5.4*
1.0 µg hLH	38.9	77.9
	±5.4	±3.6***
Large follicle		
Control	77.6	72.6
	±15.5	±12.8
10.0 µg hFSH	591.3	639.4
	±21.1	±38.6
1.0 µg hLH	830.9	810.8
	±52.2	±55.3

<sup>a</sup> Data are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 60 min with hFSH or with hLH in the absence or presence of 0.2 mM MIX and the incubation medium cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent).

\**p* < 0.05.

\*\**p* < 0.01.

\*\*\**p* < 0.001.

that in 20-min incubations of porcine GC from medium-sized follicles, addition of 3.0 mM aminophylline to incubation medium containing either FSH or LH significantly increased the concentration of intracellular cAMP when compared to the effect of FSH or LH alone. The difference in these findings and the results observed in the present studies could be due to differences in the effect of the two inhibitors on GC phosphodiesterase activity; it is possible that aminophylline has a synergistic effect with the gonadotropins in stimulating cAMP production. In another series of experiments, addition of theophylline alone without gonadotropins to incubations of isolated prepubertal rat ovaries resulted in

a stimulation of cAMP accumulation significantly above control levels in both the tissue and in the incubation medium (16). The effects of theophylline could have been due to the indirectly mediated inhibitory influence upon protein synthesis or due to a direct inhibition of phosphodiesterase (17).

If cAMP is protected from the hydrolytic action of phosphodiesterase by subcellular compartmentalization in GC, inhibition of the degradative enzymatic activity by methylxanthine would not be significantly apparent. Cheung (18) has shown that cAMP bound to the protein kinase regulatory subunit is not susceptible to phosphodiesterase activity and only is degraded when dissociated from the protein. It was concluded that the rate of hydrolysis of cAMP is governed by its rate of dissociation from the protein kinase regulatory subunit. In the present studies it is possible that the lack of a significant potentiating effect of methylxanthine upon gonadotropin stimulation of intracellular cAMP accumulation could have resulted from cAMP being bound to the protein kinase regulatory subunit during the time intervals examined. Means *et al.* (19, 20) observed that when testis were incubated for 1 hr with FSH, the protein kinase remained maximally active following an additional 2 hr of incubation without the gonadotropin present. Similar compartmentalization of intracellular cAMP may occur in porcine GC and explain the lack of a significant potentiating response of the phosphodiesterase inhibitor.

The finding that methylxanthine has a significant potentiating effect upon cAMP content in the incubation medium in response to either hFSH or hLH stimulation could be due to the presence of plasma membrane fragments in the incubation medium which makes the phosphodiesterase more accessible to the inhibitory action of MIX. Alternatively, it is possible that an extracellular phosphodiesterase may exist and have a role in the degradation of cAMP released from the GC. It is apparent from these and previous studies (4) that significant concentrations of cAMP are released extracellularly by porcine GC in response to the stimulatory action of the gonadotropins. Enzymatic degradation of extracellular cAMP has been reported for

ions of prepubertal rat ovaries using appearance of labeled cAMP as well as detection of labeled products of cAMP degradation, indicating that cAMP released into incubation medium was undergoing extracellular degradation by a phosphodiesterase (21).

Factors influencing the intracellular localization of cAMP, extracellular release, plasma membrane permeability, and metabolism of cAMP in porcine GC require more definitive studies before the questions posed can be answered.

It is possible that the MIX may have side effects other than inhibition of phosphodiesterase.

**Study.** In order to examine a possible role of phosphodiesterase in mediation of the effects of LH and FSH upon granulosa cell levels, porcine (GC) from small (1–2 mm), medium (3–5 mm), and large (6–12 mm) follicles were incubated with human hFSH and LH (hLH) for 30 and 60 min in the absence or presence of 3-isobutyl-1-methylxanthine (MIX), a potent phosphodiesterase inhibitor. Subsequently, the intracellular and incubation medium cAMP concentrations were determined by a protein binding assay. During a 30-min incubation, 10  $\mu$ g of MIX alone brought about an 11-fold, 5-fold, and 4-fold increase in intracellular cAMP in small, medium, and large follicles, respectively. Addition of 0.2 mM MIX exerted a nonsignificant ( $p > 0.05$ ) potentiating effect upon hFSH stimulation of intracellular cAMP accumulation in cells obtained from the three types of follicles. In the case of cells obtained from small and medium follicles, addition of 0.2 mM MIX in the presence of 10  $\mu$ g/ml hFSH or 10  $\mu$ g/ml hLH led to 41 to 69% potentiation ( $p < 0.001$ ) of the effect of the FSH and LH on cAMP accumulation in the incubation medium. This was evident after a 30-min incubation period. In the case of cells obtained from large follicles, addition of 0.2 mM MIX had a nonsignificant potentiating effect ( $p > 0.05$ ) on either hFSH or hLH stimulation of cAMP accumulation in the incubation medium.

It may be concluded that probably there

are low levels of intracellular phosphodiesterase in porcine granulosa cells and that gonadotropins act to stimulate the generation of cAMP rather than alter the rate of destruction of cAMP. The findings support the existence of an extracellular phosphodiesterase which may act to regulate or modulate the extracellular levels of cyclic AMP.

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## Effect of Big and Little Gastrins on Pancreatic and Gastric Secretion (40322)

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Gastrin exists in several molecular forms, of which, big gastrin (G34) and little gastrin (G17), account for most of the gastrin in circulation (1). The molar concentration of G34 in blood plasma is about twice that of G17. Infusion of equimolar doses of G34 and G17 produces approximately equal gastric acid secretory responses despite the fact that the molar blood concentrations of G17 are about five to seven times greater than those of G34, reflecting the slower removal of G34 from circulation.

It is not known whether the different molecular forms of gastrin have different relative potencies for various target organs. To explore this question we studied simultaneous gastric acid and pancreatic protein secretion responses to G34 and G17 in dogs with gastric and pancreatic fistulas. The dog is a suitable animal for such studies since in this species the doses of gastrin needed to stimulate pancreatic protein secretion and gastric acid secretion are in the same range (2).

**Materials and Methods.** Natural human little gastrin (G17-I) and natural porcine big gastrin (G34-II) were kindly provided by Professor R. A. Gregory and Doctor J. H. Tracy, University of Liverpool, England. Cholecystokinin (CCK), 20% pure, was obtained from the G.I.H. Research Unit, Karolinska Institutet, Stockholm, Sweden.

**Subjects.** Four dogs weighing 20 to 24 kg were prepared with a Thomas gastric fistula and a pancreatic fistula (PF) by a modified Herrera technique (3). Studies were begun no sooner than 4 weeks after surgery. Food and water were withheld for 18 hr before each test. The interval between tests was at least 48 hr.

**Infusions.** NaCl (0.15 M) was infused continuously into a leg vein at 30 ml hr<sup>-1</sup>. Gastrin peptides were added to the saline infusion to give the required doses (25, 50, 100, 200, 400, 800, and 1600 pmol kg<sup>-1</sup> hr<sup>-1</sup> of G34 and 53, 106, 213, 425, and 851 pmol kg<sup>-1</sup> hr<sup>-1</sup> of G17). Each dose was given

during 45 min starting with the lowest dose and doubling it until the highest dose was given. Gastric and pancreatic juices were collected continuously and separated into 15-min samples. Volumes were measured to the nearest 0.1 ml. Acid concentration was determined by titrating 0.2-ml samples with 0.2 M NaOH to pH 7 on an automatic titrator (Radiometer, Copenhagen). Total protein concentration was measured spectrophotometrically at 280 nm, using bovine serum albumin as standard. The responses were expressed as the mean of the last two 15-min collections from each dose. Two tests were done with each stimulant in each of three dogs and a fourth dog had one test with each stimulant. Basals were subtracted from each 15-min sample, and results of the two tests in each dog were averaged. Before averaging, the square root of acid output was computed and used in all analyses to make variances more uniform and straighten out the response curves.

**Results.** G34-II and G17-I were found to be approximately equipotent in stimulating gastric acid secretion (Fig. 1), confirming earlier studies (6). The relative potency of G17 with respect to G34 was 0.7 with 95% limits of 0.4 to 1.5 using doses 100, 200, and 400 for G17 and 50, 100, and 200 for G34. G34-II and G17-I appeared to differ from each other in potency in stimulating pancreatic protein secretion (Fig. 2). Relative potency of G17 with respect to G34 was about 0.3 to 0.4, depending on the doses used, with limits of about 0.1 to 0.6. The response to CCK is shown for comparison. CCK did not stimulate acid secretion. Relative potency of CCK to G17 was 1.5 (0.99 to 2.4) and to G34 was 0.5 (0.3 to 0.7).

The data do not, however, show a significant difference in selectivity for gastric acid and pancreatic protein secretion between G17 and G34. Comparison of the relative potency of G17 to G34 for acid secretion to that for protein secretion was made by computing



potency of G17 to G34 for each dog separately for acid and for protein. The mean differences  $\pm$  SE for relative potency for acid secretion minus relative potency for protein secretion were  $0.49 \pm 0.29$  and  $0.58 \pm 0.30$  depending on the G34 doses used for estimating protein potency. These differences were not significant by paired *t* test. As a further comparison, we computed the equation:  $\text{protein} = a + b (\text{acid})^1$  for each dog for each test. The slopes were similar for G17 and G34. Figure 3 shows means for pancreatic protein response plotted against gastric acid response.

**Discussion.** These studies show that the potency of G34 relative to G17 is not significantly different for gastric acid and pan-

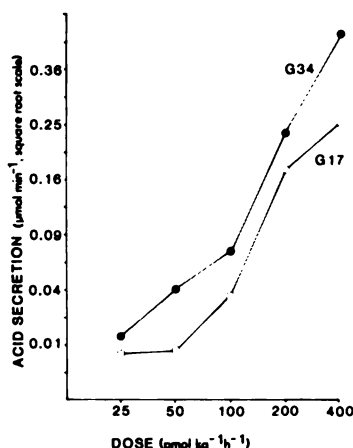


FIG. 1. Acid secretion in response to graded doses of G34 and G17.

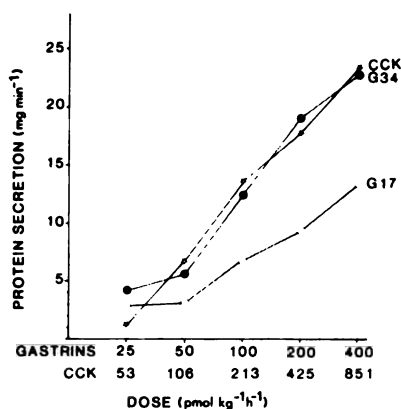


FIG. 2. Pancreatic protein secretion in response to graded doses of G34, G17, and CCK.

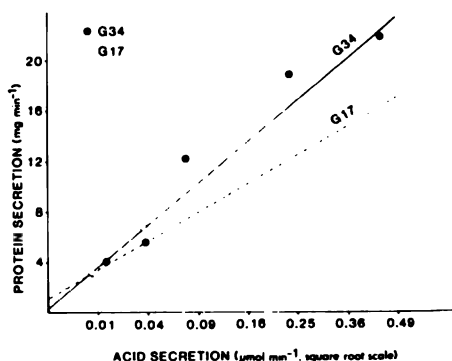


FIG. 3. Linear regression of protein secretion on acid secretion.

creatic protein secretion, indicating that one of these gastrins is not more selective than the other for these targets. Although the present results do not show a large difference in selectivity, further studies with other gastrins or other targets or in other species might reveal such differences.

**Summary.** In dogs with gastric and pancreatic fistulas the potency of porcine big gastrin (G34-II) relative to human little gastrin (G17-I) was not significantly different for stimulation of gastric acid and pancreatic protein secretion.

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## Differential Centrifugation Studies of Guinea Pig Lung Proteases (40323)

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The literature has indicated the presence of several cathepsins in lung tissue. In whole organ preparations, Otto (1) reported the presence of several rat organs, including lung, containing cathepsins B1 and B2. McDonald and others (2), using aqueous extracts of a variety of rat tissues and employing highly purified synthetic substrates, reported that lung contained dipeptidylpeptidase I (cathepsin II, III, and IV. Finally, cathepsin D was isolated and purified from extracts of homogenized rabbit and beef lung (3). Very little is known about the properties of proteolytic enzymes in this tissue, or their distribution among the subcellular fractions of lung. A preliminary study of lung (4) indicated that the subcellular fractions prepared were heterogeneous, that enzyme markers were widely distributed among the fractions, and that classical lysosomal enzymes appeared to be distributed differently in lung than in liver. With lung containing several different kinds of cells, one might expect a heterogeneity of organellar enzymes consequently a wider distribution of enzymes in particles of varying size. Interest in proteolytic enzymes in lung, normal (5, 6) and pathological (7) conditions prompted us to examine the distribution of 18 enzymes among five subcellular fractions prepared by differential pelleting of marker enzymes and eleven proteases. Proteases were examined to lay a basis for a detailed examination of lung lysosomal and lysosomal proteases and pepti-

then suspended in cold 0.25 M sucrose solution (pH 7.2). The suspended material was disrupted by brief homogenization, achieved by five up-and-down strokes of a motor-driven (1000 rpm) Potter-Elvehjem homogenizer (clearance, 4-6  $\mu$ m). This homogenate protocol consistently resulted in high yields of intact lysosomes. The homogenate was brought to 10% (w/v) with 0.25 M sucrose and was filtered through cheesecloth prior to centrifugation. Fractionation of the homogenates was achieved by 5-fraction differential centrifugation following the procedure for liver (8) without modification. These fractions were: nuclear, N (510g  $\times$  10 min); heavy mitochondrial, M (10,000g  $\times$  5 min); light mitochondrial, L (40,000g  $\times$  10 min); microsomal, P (100,000g  $\times$  45 min); and soluble, S (nonsedimentable). Total activity of 18 enzymes was investigated in each of these fractions after treatment with Triton X-100 (Sigma). The overall concentration of Triton X-100 was 0.2% (w/v). It was used to release membrane-bound enzyme activities. This low level of Triton did not affect any of the enzyme assays.

**Enzyme analysis.** All concentrations given are final concentrations in the assay mixture. Cytochrome oxidase and cathepsin D were assayed as described by Canonico and Bird (9). Lactate dehydrogenase was detected using Sigma Kit 500. *N*-Acetyl- $\beta$ -glucosaminidase was detected using the (0.02 M) *p*-nitrophenyl derivative (Sigma) in 0.1 M acetate buffer, pH 5.0. The reaction was stopped with 1.25 N NaOH and filtered (Whatman No. 42), and the absorbance was read at 440 nm on a Gilford spectrophotometer. Acid *p*-nitrophenylphosphatase was assayed as described by Bosmann and Hemsworth (10). Alkaline *p*-nitrophenylphosphatase was determined by the procedure of Garen and Levinthal (11) except that the pH was held at 8.8 (where the color is somewhat more intense) rather than at 8.0. Succinate dehydro-

**Materials and methods. Disruption and subfractionation.** Lungs were obtained from a short-haired, outbred guinea pig of a local colony. The lungs were perfused (pulmonary artery) with 200 to 300 ml saline to remove all blood, minced, and

<sup>1</sup>Supported in part by a grant from The National Institutes of Health (HL 16920).

genase was measured using the method of Pennington (12) in which the dye 2-(*p*-iodophenyl)-3-*p*-nitrophenyl)-5-phenyl-tetrazolium was reduced by succinate to produce formazan which was extracted into ethyl acetate and read at 490 nm. Glucose 6-phosphatase was determined by the method of Nordlie and Arion (13) using the sodium cacodylate buffer, pH 6.5. Inorganic phosphorus was determined by the method of Chen *et al.* (14). Cathepsin A was measured using the method of Iodice *et al.* (15) with *N*-carbobenzoxy- $\alpha$ -glutamyl-L-tyrosine (Cyclo Chemical Co., Los Angeles, California)<sup>2</sup> as substrate. The rate of production of free amino groups was monitored with the ninhydrin reagent of Moore and Stein (16). To analyze the cathepsin B1, the method of Barrett (17) was employed. Dipeptidylpeptidases I, II, III, and IV were determined using the method of McDonald *et al.* (18). The substrates used for the fluorimetric assays were as follows: dipeptidylpeptidase I, 0.1 mM Gly-Arg- $\beta$ -naphthylamide (2) in 5 mM NaCl-7.5 mM 2-mercaptoethanol-70 mM sodium succinate, pH 5.0; dipeptidylpeptidase II, 0.2 mM Lys-Ala- $\beta$ -naphthylamide in 10 mM 3,3-dimethylglutaric acid, pH 5.5; dipeptidylpeptidase III, 0.03 mM Arg- $\beta$ -naphthylamide in 62.5 mM Tris-HCl, pH 9.0; dipeptidylpeptidase IV, 0.17 mM Gly-Pro- $\beta$ -naphthylamide in 20 mM Tris-HCl, pH 7.8. Calibration was carried out with known standards of  $\beta$ -naphthylamine. All  $\beta$ -naphthylamides were purchased from Bachem (Torrance, California). For the analysis of elastolytic esterase, the method of Visser and Blout (19) was used. In this procedure, 0.33 mM *p*-nitrophenyl *N*-tert-butyloxycarbonyl-L-alanate (Sigma) was used as substrate in 0.05 M sodium phosphate-3% acetonitril, pH 6.5. Dipeptidase was assayed using the titrimetric assay of Bryce and Rabin (20). Glycyl-L-leucine was used as the substrate. A radiometer titrigraph Type SBR 2c was used to keep the pH constant at 8.4 by adding standardized acid. Neutral and alkaline protease activities were measured on 1% heat-denatured casein solutions at pH 7.0 and 8.5, respectively, similar to the method of

Kunitz (21). After 30 min, 10% trichloroacetic acid was used to precipitate proteins and large peptide fragments. The absorbance of the supernatant at 280 nm was used as an indication of protease activity.

Protein was determined by the Biuret method of Gornall *et al.* (22) using bovine serum albumin Fraction V (Sigma) as standard.

**Presentation of results.** To simplify construction of tables and graphs, the following symbols were used: N = nuclear fraction; M = heavy mitochondrial fraction; L = light mitochondrial or lysosomal fraction; P = microsomal fraction; S = final supernatant or cytoplasmic fraction.

The percentage of an enzyme in any one fraction was determined by dividing the activity in that fraction by the total activity obtained in the five fractions  $\times 100$ . The percentage recovery was determined by dividing the sum of an enzyme's activity in the five fractions, N, M, L, P, and S, by the activity determined on a sample of homogenate prior to centrifugation,  $\times 100$ .

The relative specific activity in each fraction was obtained as follows: percentage of total activity/percentage of total protein  $\times 10$ , according to de Duve *et al.* (8).

The distributions of the enzymes' activities after differential centrifugation are presented by plotting the mean relative specific activity against the protein content of each fraction. The area of each block represents the percentage of the total activity recovered in that fraction, and the height corresponds to the degree of purification achieved (8).

Enzyme specific activities are presented in milliunits per milligram of protein where 1 unit equals 1  $\mu$ mole of substrate hydrolyzed, or 1 unit of absorbance released at 280 nm, per minute at 37°C. The units for cytochrome oxidase are calculated according to Cooperstein and Lazarow (23).

**Results. Enzyme distribution following differential centrifugation.** The distribution of 18 enzymes and of the lung protein following differential centrifugation are presented in Table I, along with the percentage of each enzyme recovered. Despite the heterogeneity of the lung cell populations, the distribution recorded for the various enzymes paralleled that found in liver. For example, the major

<sup>2</sup> Cyclo Chemical Company's inventory has been purchased by Vega-Fox Biochemicals, Tuscon, Arizona.

TABLE I. PERCENTAGE OF TOTAL ACTIVITY IN TISSUE FRACTIONS.<sup>a</sup>

Enzymes	Fraction					Percentage enzyme recovered
	N	M	L	P	S	
e oxidase (1)	17.3	65.6	16.4	0.7	0	85.5
lehydrogenase (3)	12.1 ± 6.8	39.2 ± 22.4	25.5 ± 15.6	12.7 ± 12.1	10.5 ± 12.6	121.7 ± 48.3
ydrogenase (3)	8.6 ± 6.2	3.7 ± 3.4	3.1 ± 0.6	7.7 ± 1.9	76.9 ± 8.1	79.2 ± 8.6
-glucosaminidase (1)	32.0	32.4	15.4	4.6	15.4	93.4
ophenylphosphatase (3)	22.6 ± 10.9	14.5 ± 5.7	21.5 ± 5.5	19.8 ± 5.3	21.5 ± 1.8	96.0 ± 6.4
nitrophenylphosphatase	4.8 ± 5.1	13.6 ± 5.7	20.9 ± 4.5	29.3 ± 5.6	31.1 ± 9.2	69.9 ± 31.9
phosphatase (3)	13.2 ± 6.0	9.8 ± 4.1	18.3 ± 4.8	25.8 ± 1.7	32.8 ± 8.6	198.6 ± 131.0
A (3)	19.0 ± 13.6	5.2 ± 5.2	31.6 ± 14.1	4.6 ± 6.3	39.7 ± 3.7	62.3 ± 24.2
B1 (3)	24.5 ± 13.9	15.2 ± 21.0	39.5 ± 24.4	3.5 ± 4.8	17.2 ± 8.4	362.5 ± 417.7
D (3)	17.5 ± 4.8	20.5 ± 5.8	20.8 ± 5.7	7.6 ± 2.8	33.6 ± 4.4	142.2 ± 29.9
peptidase I (3)	13.9 ± 17.1	8.9 ± 1.6	25.6 ± 4.8	3.4 ± 2.9	48.2 ± 12.8	68.4 ± 12.8
peptidase II (3)	18.7 ± 8.8	24.1 ± 9.0	20.2 ± 5.6	4.2 ± 3.2	32.7 ± 9.9	114.5 ± 33.6
peptidase III (3)	1.8 ± 1.1	5.9 ± 9.0	4.4 ± 4.7	2.6 ± 0.7	85.3 ± 15.2	147.5 ± 12.2
peptidase IV (3)	16.6 ± 14.9	9.4 ± 3.8	22.1 ± 4.9	33.5 ± 13.8	18.4 ± 3.3	113.2 ± 16.2
esterase (3)	11.9 ± 7.3	9.2 ± 3.7	15.5 ± 3.4	10.5 ± 2.7	52.9 ± 6.5	99.2 ± 15.8
tease (3)	3.9 ± 3.8	5.5 ± 3.8	11.3 ± 2.0	29.7 ± 13.1	49.5 ± 21.5	139.2 ± 30.2
otase (3)	2.8 ± 3.9	5.3 ± 4.6	11.4 ± 1.0	31.1 ± 13.2	49.4 ± 20.3	151.2 ± 16.3
e (1)	1.3	0.7	1.3	1.8	95.0	118.1
	21.3 ± 12.2	13.2 ± 3.3	9.9 ± 4.0	11.5 ± 4.5	44.2 ± 7.2	104.5 ± 4.7

<sup>a</sup> indicate the mean percentage ± the standard deviation of the mean. Numbers in parentheses indicate the number of experiments. The enzyme activity and percentage enzyme recovered were calculated as described in the section under Experimental.

the activities of both cytochrome oxidase and succinate dehydrogenase is found in heavy mitochondrial fraction, and of lactate dehydrogenase in the supernatant fractions expected.

percentage recovery of nine enzymes: cytochrome oxidase, succinate dehydrogenase, succinate dehydrogenase, *N*-acetyl- $\beta$ -glucosaminidase, acid-*p*-nitrophenylphosphatase, dipeptidylpeptidase II and IV, elastolytic esterase, and dipeptidase) and of protein food (i.e., 79–122%); the recoveries of enzymes (alkaline *p*-nitrophenylphosphatase, cathepsin A, and dipeptidylpeptidase) were low (62–70%) whereas six enzymes (glucose 6-phosphatase, cathepsins B1, dipeptidylpeptidase III, and neutral alkaline protease) showed significantly higher total activity in the sum of the fractions in the whole homogenate (Table I). It is possible that fractionation removed an inhibitor of these enzymes and allowed better expression of total activity in the fractions. This has already been demonstrated in our laboratory, where the addition of a small aliquot of the supernatant fraction to the light mitochondrial fraction strongly increased cathepsin B1 activity, as measured by the hydrolysis of benzoyl-arginyl- $\beta$ -naphthylamide (24).

**Relative specific activities.** The relative specific activity of each enzyme in each of the

five tissue fractions is presented in Table II. These values were plotted vs the percentage protein in each fraction to give the graphs which are presented in Fig. 1.

Cytochrome oxidase and succinate dehydrogenase, two mitochondrial markers, were enriched in the heavy mitochondrial fraction, M, and to a lesser extent in the light mitochondrial fraction, L.

*N*-Acetyl- $\beta$ -glucosaminidase, acid *p*-nitrophenylphosphatase, dipeptidylpeptidase I, dipeptidylpeptidase II, cathepsin A, cathepsin B1, cathepsin D, and elastolytic esterase all showed greatest enrichment in the light mitochondrial fraction, L. Among these enzymes there appeared to be two separate patterns of distribution. Cathepsin A, cathepsin B1, and dipeptidylpeptidase I appeared to distribute so that the light mitochondrial fraction was greatly enriched over the neighboring fractions. On the other hand, *N*-acetyl- $\beta$ -glucosaminidase, cathepsin D, dipeptidylpeptidase II, acid *p*-nitrophenylphosphatase, and elastolytic esterase distributed throughout the fractions such that the light mitochondrial fraction, L, was only slightly enriched over the neighboring fractions. In this second class of enzymes, the distribution throughout the fractions seemed to be broader than the first class.

The microsomal fraction, P, was enriched in glucose 6-phosphatase, alkaline *p*-nitro-

TABLE II. RELATIVE SPECIFIC ACTIVITIES IN TISSUE FRACTIONS.\*

Enzyme	Fraction				
	N	M	L	P	S
Cytochrome oxidase (1)	0.7	5.2	3.9	0.1	0
Succinate dehydrogenase (3)	0.9 ± 0.8	3.8 ± 1.4	3.5 ± 1.1	1.5 ± 1.2	0.3 ± 0.4
Lactate dehydrogenase (3)	1.1 ± 0.3	0.6 ± 0.2	1.0 ± 0.3	2.0 ± 0.3	5.1 ± 0.6
<i>N</i> -Acetyl- $\beta$ -glucosaminidase (1)	1.3	2.8	4.2	1.0	0.6
Acid <i>p</i> -nitrophenylphosphatase (3)	1.6 ± 0.7	1.7 ± 0.8	3.3 ± 0.4	2.6 ± 0.4	0.7 ± .03
Alkaline <i>p</i> -nitrophenylphosphatase (3)	0.5 ± 0.5	1.4 ± 0.2	3.2 ± 0.4	3.9 ± 0.5	1.0 ± 0.3
Glucose 6-phosphatase (3)	1.1 ± 0.6	1.3 ± 0.8	2.9 ± 0.2	3.7 ± 0.9	1.1 ± 0.3
Cathepsin A (3)	1.7 ± 1.6	0.6 ± 0.5	5.5 ± 0.8	0.6 ± 0.7	1.6 ± 0.5
Cathepsin B1 (3)	2.0 ± 1.7	1.2 ± 1.5	5.7 ± 1.7	0.4 ± 0.5	0.6 ± 0.3
Cathepsin D (3)	1.5 ± 0.4	2.6 ± 1.0	3.6 ± 0.5	1.1 ± 0.4	1.2 ± 0.3
Dipeptidylpeptidase I (3)	0.7 ± 0.5	1.4 ± 0.7	5.2 ± 0.8	0.5 ± 0.3	2.1 ± 0.8
Dipeptidylpeptidase II (3)	1.6 ± 0.4	3.1 ± 0.7	3.6 ± 0.5	0.6 ± 0.3	1.2 ± 0.3
Dipeptidylpeptidase III (3)	0.3 ± 0.3	0.8 ± 0.9	1.5 ± 1.6	0.8 ± 0.2	6.6 ± 2.6
Dipeptidylpeptidase IV (3)	1.0 ± 0.2	1.0 ± 0.1	3.4 ± 0.7	5.1 ± 2.4	0.6 ± 0.2
Elastolytic esterase (3)	1.1 ± 0.4	1.4 ± 0.8	3.3 ± 0.8	1.9 ± 0.5	2.3 ± 0.5
Neutral protease (3)	0.5 ± 0.5	0.8 ± 0.7	2.1 ± 0.5	4.5 ± 0.7	2.0 ± 1.1
Alkaline protease (3)	0.4 ± 0.5	0.9 ± 0.8	2.2 ± 0.6	4.6 ± 0.7	2.0 ± 0.9
Dipeptidase (1)	0.5	0.3	0.6	0.5	8.1

\* Values are the mean relative specific activity  $\pm$  standard deviation of the mean. Numbers in parentheses are the number of experiments.

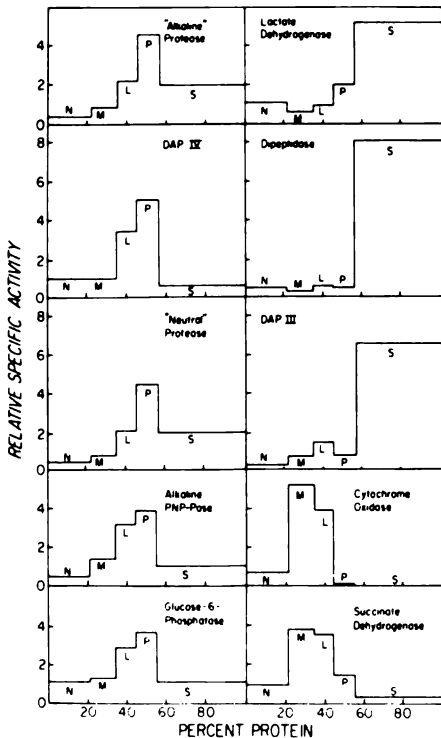


FIG. 1. Distribution patterns of enzymes after differential centrifugation. Fractions are N, nuclear; M, heavy mitochondrial; L, light mitochondrial; P, microsomal; and S, nonsedimentable. Enzyme abbreviations are

phenylphosphatase, neutral protease, dipeptidylpeptidase IV, and alkaline protease. For these enzymes considerable activity was also found in the light mitochondrial fraction.

Three enzymatic activities were found in the cytosol: lactate dehydrogenase, dipeptidase, and dipeptidylpeptidase III. The specific activities for the 18 enzymes in guinea pig lung are presented in Table III.

**Discussion.** This study of differential centrifugation, combined with the biochemical analysis of marker enzymes, satisfies the criteria of de Duve *et al.*, (8) for separation of organelles. The fact that the mitochondrial enzymes, cytochrome oxidase and succinate dehydrogenase, the lysosomal enzyme, *N*-acetyl- $\beta$ -glucosaminidase, the microsomal enzyme, glucose 6-phosphatase, and the cytosol enzyme, lactate dehydrogenase, were enriched in the fractions M, L, P, and S, respectively, indicated that the experimental procedure employed was capable of resolving to some degree the designated subcellular organelles. The somewhat broad distributions observed with these markers indicated that the fractions were heterogeneous in the organelles they contained. This was confirmed

DAP, dipeptidylaminopeptidase or dipeptidylpeptidase; PNP-Pase, *p*-nitrophenylphosphatase.

III. SPECIFIC ACTIVITIES OF VARIOUS LUNG ENZYMES.<sup>a</sup>

ne oxidase (5)	50.2 ± 7.8
dehydrogenase (4)	12.3 ± 2.2
A (4)	46.3 ± 20.4
B1 (4)	3.4 ± 0.6
lpeptidase I (2)	10.8
D (5)	11.7 ± 2.7
esterase (4)	31.7 ± 2.0
lpeptidase II (2)	1.2
β-glucosaminidase (5)	5.2 ± 1.7
rophenylphosphatase (5)	5.3 ± 1.7
-nitrophenylphosphatase	12.0
phosphatase (2)	1.6
lpeptidase IV (2)	2.9
rotease (2)	4.0
otease (2)	5.1
lpeptidase III (2)	3.1
se (1)	1086.0
hydrogenase (2)	402.0

Values (obtained with the whole homogenate) units per milligram of protein ± the standard error mean. The numbers in parentheses are the number of experiments.

Microscopic examination. The broad distribution seen in guinea pig lung were also seen in rabbit lung (4).

Other enzymes were used as secondary markers. Acid *p*-nitrophenylphosphatase served as a lysosomal marker in spite of the fact that isoenzymes of the true acid phosphatase exist in different parts of the cell. While the distribution of *p*-nitrophenylphosphatase activity was broad, it did correspond to that of a lysosomal enzyme. Alkaline *p*-nitrophenylphosphatase was also used as a secondary marker of the "microsomal" fraction since this enzyme has generally been considered as being a component of the plasma membrane (26). Fragmental plasma membranes of rat kidney (27) and liver (28) have been found to sediment with the microsomal fraction.

Distribution of the dipeptidylpeptidase (labeled dipeptidylaminopeptidase) enzymes in lung parallels their distributions in other tissues. Dipeptidylpeptidase I (or dipeptidylaminopeptidase I or cathepsin C) has been localized in rat liver (2) and bovine kidney (2). A cytosol distribution was noted for dipeptidylpeptidase III from bovine kidney (2). Dipeptidylpeptidase IV has been shown to have a microsomal distribution in porcine kidney (2) and rat liver (2). The number of proteolytic enzymes has been

localized in subcellular fractions in tissues other than lung, largely using the technique of differential centrifugation. Cathepsin A and another carboxypeptidase-like enzyme appeared to be found in the heavy mitochondrial fraction (29); cathepsins B (29) and D (30) were lysosomal in origin; and di- and tripeptidases in different tissues have been variously reported as being in the supernatant (29) and in the microsomal fractions (31, 32). Lung cathepsins A, B1, and D all appeared to be lysosomal in nature. The lung dipeptidase activity was found to be clearly associated with the cytosol fraction, as assayed with Gly-L-Leu, Gly-Gly, Gly-DL-Phe, or Gly-DL-Ser. Of eight dipeptides tested with this enzyme, the most effective substrate was Gly-L-Leu, the data for which are reported here. No tripeptidase activity against Gly-Gly-Gly or L-Leu-Gly-Gly was noted.

Elastolytic esterase was determined by the rate of breakdown of a synthetic substrate, *p*-nitrophenyl *N*-tert-butyloxycarbonyl-L-alanate. The enzyme present could not be detected using the orcein-elastin assay (33). The failure to react with the latter substrate could have been due to extremely low levels of elastase or to the fact that this enzyme was not a true elastase. Such an enzyme has been recently characterized from human pancreas (34). We have, therefore, chosen to call the enzyme measured, elastolytic esterase. This enzyme was enriched most in the light mitochondrial fraction, L, but was also present in the cytosol in sizeable quantities. The possibilities of a dual location of the same enzyme or of two different enzymes remain for consideration.

Neutral and alkaline proteases distributed with the microsomal enzyme markers. The similarity of distribution and the method of assay of the two proteases would leave open the possibility that the same enzyme is being measured at two different pH values.

The distribution of enzymes noted in this work does not differ significantly from the distributions of similar enzymes in other tissues. Further work performed on guinea pig lung using isopycnic-zonal centrifugation to obtain better resolution of fractions will be reported.

**Summary.** Five subcellular fractions were isolated from guinea pig lung homogenates by differential centrifugation. These fractions

were defined biochemically by the analysis of 18 enzymes representing different subcellular compartments. Succinate dehydrogenase and cytochrome oxidase distributed with the heavy mitochondrial fraction, while *N*-acetyl- $\beta$ -glucosaminidase, acid *p*-nitrophenylphosphatase, cathepsins A, B1, and D, dipeptidylpeptidases I and II, and elastolytic esterase distributed with the light mitochondrial fraction. Alkaline *p*-nitrophenylphosphatase, glucose 6-phosphatase, dipeptidylpeptidase IV, neutral protease, and alkaline protease all demonstrated a "microsomal" enrichment. In the cytosol were found lactate dehydrogenase, dipeptidylpeptidase III, and a dipeptidase. The lung subcellular fractions were heterogeneous with cross-contamination between the heavy mitochondrial, light mitochondrial, and "microsomal" fractions. The enzyme distributions noted were similar to those found in other tissues.

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## Fluctuations of Human Pancreatic Polypeptide in Plasma: Effect of Normal Food Ingestion and Fasting<sup>1</sup> (40324)

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Secretion of human pancreatic polypeptide (hPP) is stimulated by food ingestion. Since this response persists for several days, it could be predicted that under the dietary habit of three meals a day hPP would be elevated above fasting levels during most of the daytime. Thus, we examined the daily fluctuations of circulating hPP in normal individuals subjected to a meal schedule and in an inverse schedule, i.e., during prolonged fasting. In this study, the effect of the ingestion of a low-bulky meal as well as tap water on hPP secretion was examined.

**Subjects and methods.** Healthy, nonobese subjects participated in this study. Their ages ranged from 20 to 24 years. Informed consent was obtained. In a group of seven male volunteers hPP plasma levels were measured at intervals from 8:30 to 24:00 hr while following meal schedule: breakfast at 8:30 hr (a cup of coffee with milk and two slices of toast); lunch at 13:00 hr (300 g of boiled chicken, 200 g of grilled beef, and one pear); and dinner at 20:00 hr (vegetable salad, 200 g of chicken, 50 g of white bread, and one apple). In a second group of 12 volunteers (three males and four females) fasting was maintained for 84 hr. They received water and 40 mEq of K<sup>+</sup> daily. Upon termination of the experiments, a body weight of 65.5 ± 0.2 kg was recorded. Blood samples were obtained 12, 18, 24, 36, 42, 48, 60, and 84 hr after the last meal, which was ingested in the evening (21:00 hr) prior to the starvation period. Volunteers were admitted to our clinical research center on the day preceding the experiments. In further experiments, six volunteers (three males and three females) were given either 400 g of vegetable salad (250 g of asparagus and 150 g of tomato) or 500 ml of tap water on two

different days. These tests were performed after an overnight fast.

The collection and processing of blood samples has been previously described (3). Plasma glucose was determined by means of a commercial glucose-oxidase preparation (Biochemica Test Combination, Boehringer Mannheim GmbH). Radioimmunoassay was used to estimate insulin (4), glucagon (5), and hPP (6). Results are expressed as means ± SEM. Differences between values were calculated for significance by paired *t* test analysis.

**Results.** Figure 1 shows the daily fluctuations of plasma hPP levels in a group of seven subjects kept on a conventional meal schedule. Mean fasting hPP concentration was 61 ± 15 pg/ml. Ingestion of each meal was followed by a sustained hPP elevation. After breakfast plasma hPP rose to 158 ± 35 pg/ml at 11:30 hr (*p* < 0.01) while lunch and dinner elicited more marked increases (551 ± 131 pg/ml at 15:00 hr, *p* < 0.01; 640 ± 153 pg/ml at 20:30 hr, *p* < 0.01, respectively). It is noteworthy that between meals circulating hPP did not return to basal values. As expected, following each meal the concentrations of glucose and insulin in plasma increased in a parallel fashion.

In view of the apparent association of hPP secretion with the consumption of food, we tested the effect of a low-calorie, bulky meal on plasma hPP (Fig. 2). This meal elicited a sixfold increase of hPP concentration with only a small rise of plasma insulin and glucose. The ingestion of even 500 ml of tap water (Fig. 3) more than doubled the levels of circulating hPP.

In Fig. 4 are depicted the mean hPP, glucagon, insulin, and glucose plasma levels for a group of 12 volunteers subjected to 84 hr of fasting. Basal (after a 12-hr overnight fast) hPP concentration was 61 ± 16 pg/ml. Prolonging of fasting resulted in an increase of circulating hPP, which became statistically

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significant 24 hr after the last meal ( $181 \pm 53$  pg/ml,  $p < 0.02$ ) and persisted elevated during the remainder of the experimental period. It is remarkable that for each day plasma hPP showed a distinct pattern, with a progressive rise from 9:00 to 21:00 hr and a

subsequent decline during the night. The overall curve, however, exhibited a rising trend. Finally, during fasting plasma glucose and insulin declined while glucagon rose.

**Discussion.** The foregoing data confirm the stimulatory effect of food intake on pancreatic polypeptide secretion in man. It is noteworthy that the ingestion of a fiber-rich meal as plain water provokes hPP release, suggesting that the hPP response to food represents in part a nonspecific effect, perhaps the consequence of gastric distention as pointed out by Schwartz *et al.* (7). Furthermore, one can demonstrate that under normal dietary conditions, the successive postprandial circulating hPP maintain its levels at a high value throughout the daytime. The physiological role of pancreatic polypeptide remains enigmatic, it is not clear whether it has the category of a digestive hormone since the administration of the bovine extract in dogs modifies gastric and pancreatic secretion as well as gastrointestinal motility (8). Contextually, the persistence of plasma hPP may be thought of as a tonic influence on some of these functions. On this basis, in conditions of food deprivation a decrease of circulating hPP

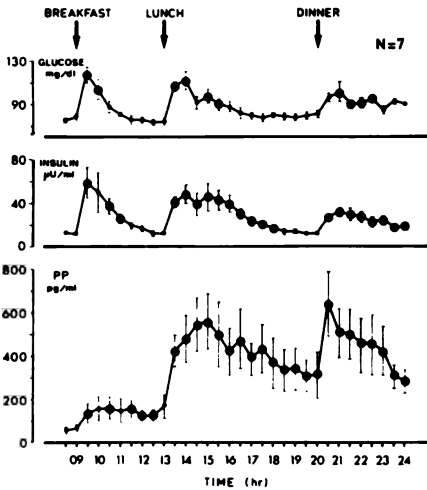


FIG. 1. Daily fluctuations of plasma pancreatic polypeptide in normal subjects under conditions of normal food ingestion (mean  $\pm$  SEM). The large dots represent statistically significant differences from the baseline values.

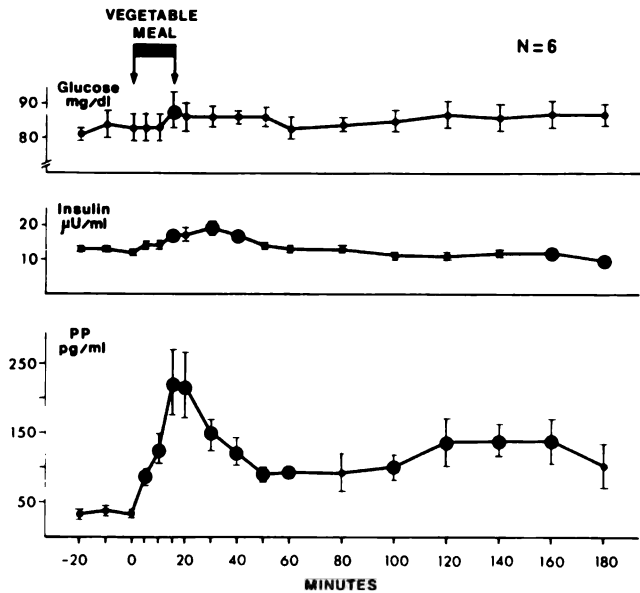
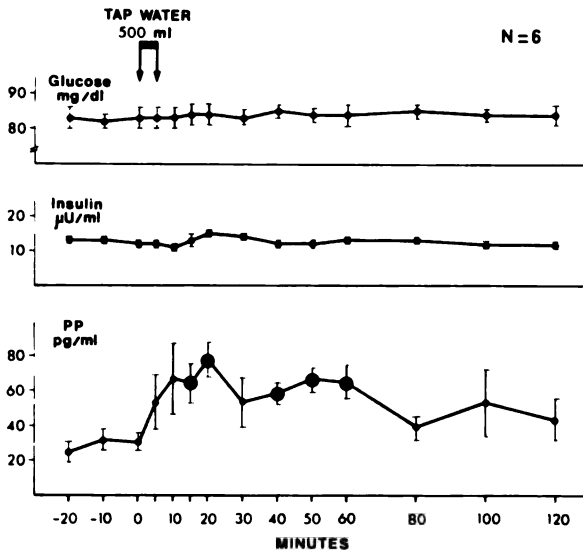
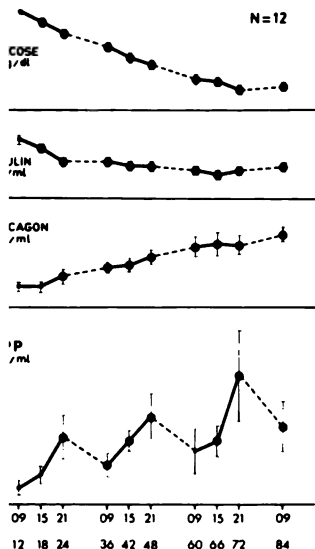


FIG. 2. Effect of ingestion of a vegetable meal on pancreatic polypeptide plasma levels in normal subjects (mean  $\pm$  SEM). The large dots represent statistically significant differences from the baseline values.



Effect of tap water ingestion on pancreatic polypeptide plasma levels in normal subjects (mean  $\pm$  SEM). The asterisks represent statistically significant differences from the baseline values.



Effect of tap water ingestion on pancreatic polypeptide plasma levels during normal feeding in normal subjects (mean  $\pm$  SEM). The asterisks represent statistically significant differences from the baseline values.

as reported for gastrin (9). However, prolonged fasting resulted in a progressive rise of this factor in blood, an observation in agreement with that of Floyd and Liddle (1). Moreover, in the absence of food, plasma hPP showed circadian variations with higher concentrations in the

late evening than in the preceding and subsequent morning. A similar pattern was observed by the above-mentioned authors with determinations at 8:00 AM and 4:00 PM. In interpreting the rise of plasma hPP during fasting, the concomitant decline of glycemia should be considered, since even a modest fall of blood sugar provokes hPP secretion (1, 6). Also, as described for glucagon (10), the possibility of diminished metabolic clearance of hPP should be contemplated. However, either of these alternatives fails to explain the circadian oscillations of hPP. Current evidence indicates that parasympathetic stimulation induces hPP secretion (11, 12) and, thus, changes in vagal tone may affect circulating hPP. Accordingly, the reduction of vagal tonic activity associated with sleep (13) could be responsible for the low hPP plasma levels found in the morning. In man, during a 24-hr fast a circadian rhythm of gastric acid secretion, with greater output in the evening than in the morning has been documented (14). The relationship between this phenomenon and the parallel changes of hPP remains speculative.

In any case, the understanding of the paradoxical rise of plasma hPP in both anabolic (feeding) and catabolic (fasting) situations awaits a better knowledge of the biological activity of this putative hormone.

**Summary.** In this work we have examined the daily fluctuations of circulating hPP in normal individuals subjected to a conventional meal schedule (breakfast, lunch, and dinner) as well as during food deprivation for 84 hr. In addition, we have tested the effect of ingestion of a low-calorie, fiber-rich salad as well as 500 ml of tap water on hPP secretion.

Ingestion of each meal was followed by a sustained hPP elevation. Between meals, circulating hPP did not return to basal values. Both the vegetable meal and the water load evoked hPP release, suggesting that the hPP response to food intake is partially a nonspecific effect. In the fasted group, plasma hPP rose significantly 24 hr after the last meal and persisted elevated for the remainder of the experimental period. Moreover, in this condition hPP showed circadian variations, with higher values in the late evening than in the preceding and subsequent morning.

Since pancreatic polypeptide is suspected to possess gastrointestinal functions, its elevation in plasma throughout the daytime in conditions of normal feeding may be thought to exert a tonic influence on some digestive process. On this basis, the increase of hPP during prolonged fasting appears paradoxical and, indeed, the explanation of this phenomenon awaits a better knowledge of the biological activity of this peptide.

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## Mechanism of Prostaglandin E<sub>2</sub> Stimulation of Renin Secretion (40325)

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Renal infusion of prostaglandins or the renin precursor, arachidonic acid, has been shown to stimulate renin secretion (1), rats (2), and rabbits (3). In addition, inhibition of prostaglandin synthesis decreases endogenous renin secretion (3), renin secretion in response to hemorrhage (4), and angiotensin-stimulated renin secretion (5). In dogs, in which renin release has been blocked by methacrin, infusion of prostaglandin E<sub>2</sub> significantly increased the release of renin beyond the original control values.

The mechanism by which PGE<sub>2</sub> increases renin secretion may involve one or a combination of three factors. First, the hormone may have a direct effect upon the juxtaglomerular apparatus. Second, PGE<sub>2</sub> may activate a vascular baroreceptor mechanism by dilation of the renal vasculature (7). Third, PGE<sub>2</sub> may stimulate a tubular macula cell receptor since in addition to decreasing resistance, PGE<sub>2</sub> infusion also increases sodium excretion (6). In the present experiments, the mechanism by which PGE<sub>2</sub> increases renin secretion was evaluated by comparison of the effect of PGE<sub>2</sub> on renin secretion with the vasodilating agents acetylcholine, bradykinin, and eleodisin.

**Materials and methods.** Male mongrel dogs anesthetized with sodium pentobarbital (30 mg/kg iv). Following insertion of a cuffed tracheal tube, dogs were artificially ventilated (Harvard Apparatus, Inc.). A femoral artery and two femoral veins were cannulated for recording of arterial blood pressure, infusion of inulin (3% solution at 1 ml/min), and infusion of saline. Blood pressure was monitored with a strain gauge pressure transducer (Statham P23AA) and a direct writing oscillograph (Grass polygraph). The left kidney was exposed via a flank incision and the renal artery and vein were cannulated with polyethylene tubing. A noncannulating electromagnetic flowmeter probe (Carolina Med-

ical Electronics) was placed on the renal artery and renal blood flow was recorded on the oscillograph. Renal venous blood samples were collected by placing a curved 20-gauge needle attached to polyethylene tubing into the renal vein. A curved 22-gauge needle attached to polyethylene tubing was inserted into the renal artery distal to the flow probe for the intrarenal infusion of PGE<sub>2</sub>, acetylcholine, bradykinin, and eleodisin. Each dog was hydrated prior to the experiment with a solution containing 140 mEq/liter sodium chloride and 3.0 mEq/liter potassium chloride, infused at 5.0 ml/min until the total urine flow rate reached 0.5 to 1.5 ml/min. The infusion rate was then decreased to equal the urine flow rate. Experiments were begun 1 hr following the completion of surgery.

In each experiment, two control clearance periods of 10 min duration each were followed by the infusion of one of the vasodilating agents. The rate of infusion of the drug was adjusted to increase renal blood flow 20 to 40%. Two additional clearance periods were obtained. Systemic arterial and renal venous blood samples were collected at the midpoint of each clearance period. Drug infusion was stopped and a 30-min period ensued during which renal blood flow returned to control levels. Drug metabolism was assumed to be complete when RBF was stable again and the next control period and drug treatment were begun. The order of administration of acetylcholine, bradykinin, and prostaglandin E<sub>2</sub> was randomized throughout the experiments. Eleodisin was always administered last, due to its presumed slower rate of metabolism. The range of doses of each vasodilator used were as follows: acetylcholine, 210 to 420 ng/kg/min; bradykinin, 7 to 21 ng/kg/min; eleodisin, 15 to 32 ng/kg/min; and prostaglandin E<sub>2</sub>, 14 to 60 ng/kg/min.

**Analytical and statistical procedures.** Plasma and urine inulin concentration were

determined by the diphenylamine method described by Walser *et al.* (8). GFR was estimated by the clearance of inulin. Plasma renin concentration was determined by radioimmunoassay for the generated angiotensin I (9). Hematocrit was measured on all arterial blood samples by the micromethod. Renal plasma flow was calculated from the renal blood flow and hematocrit. Sodium and potassium concentration of both plasma and urine were determined by flame photometry and the electrolyte excretion rates were calculated. Renin secretion was calculated as the product of the renal venous-arterial renin concentration difference and renal plasma flow. Renal blood flow and renin secretion mean differences were tested by a paired *t* analysis. Sodium and potassium excretion was calculated as the percentage increase from control and treatments were compared by one-way analysis of variance. The 0.05 level of probability was used as the criterion of significance.

**Results.** Infusion of PGE<sub>2</sub> significantly increased renal blood flow (Fig. 1). The increase in renal blood flow was associated with an increase in renin secretion from a control value of  $925 \pm 327$  to  $1710 \pm 486$  ng/min (Fig. 1). Eledoisin also increased renal blood flow but did not change renin secretion (Fig. 1).

Both acetylcholine and bradykinin increased renal blood flow but neither drug affected renin secretion (Fig. 2).

Renal vasodilation with acetylcholine, bradykinin, PGE<sub>2</sub>, or eledoisin increased both sodium and potassium excretion of the treated kidney (Table I). The percentage increases following each drug were not significantly different from each other. The sodium and potassium excretion of the contralateral kidney was not affected by drug infusion. The glomerular filtration rate of the treated and untreated kidneys did not change during drug infusion. Unilateral renal vasodilation did not alter the mean systemic blood pressure.

**Discussion.** The secretion of renin may be altered by a vascular mechanism located in the afferent glomerular arteriole (7) since decreases in renal resistance stimulate the release of renin (10). The present experiments demonstrate that PGE<sub>2</sub> increased both ipsilateral renal blood flow and renin secretion (Fig. 1) while not affecting mean systemic blood pressure or contralateral renal function. Renal vasodilation due to acetylcholine, bradykinin, or eledoisin, however, did not affect renin secretion (Figs. 1 and 2). Since the increase in renal blood flow was similar following infusion of all drugs, a vascular

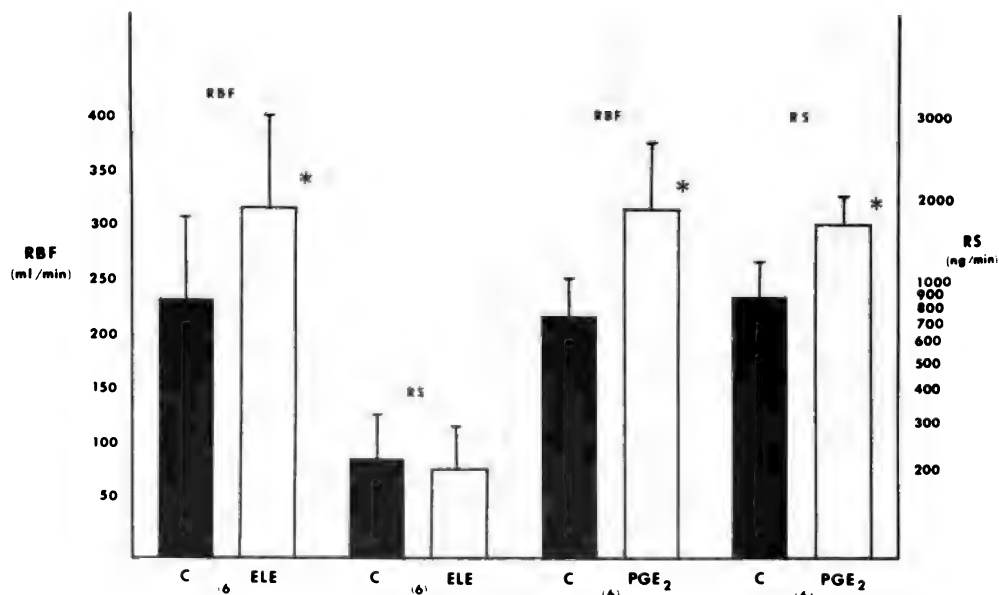


FIG. 1. Effect of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and eledoisin (Ele) on renal blood flow (RBF) and renin secretion (RS). C = control. Values are expressed as means  $\pm$  SEM. *n* = 6. \**p* < 0.05.

anism does not appear to be primarily responsible for the increase in renin secretion following PGE<sub>2</sub>.

Intrarenal infusion of bradykinin has been shown to increase renal PGE secretion (11). Therefore, bradykinin may affect renin secretion in a manner similar to that of PGE<sub>2</sub>. In the present experiments, the dose of bradykinin which increased renal blood flow approximately 20 to 40% of control was less than the dose of bradykinin previously reported to increase PGE release (11). Thus, plasma or tissue PGE<sub>2</sub> concentration increased in response to bradykinin in these experiments may not have been sufficient to elicit a response similar to that produced by infusion of PGE<sub>2</sub>.

A tubular mechanism located at the macula densa region of the distal nephron also

affects renin secretion by sensing changes in tubular sodium or chloride transport (12). Acetylcholine and PGE<sub>2</sub> have been shown to decrease proximal tubular sodium reabsorption (13). Similarly, bradykinin decreased proximal tubular sodium reabsorption by a mechanism related to vasodilation of the renal vasculature (14). The present data demonstrate that intrarenal infusion of acetylcholine, bradykinin, PGE<sub>2</sub>, or eleodisin increased sodium and potassium excretion (Table I) to a similar degree in all experiments without affecting GFR. Since changes in tubular sodium reabsorption or changes in potassium excretion following infusion of acetylcholine, bradykinin, eleodisin, or PGE<sub>2</sub> are similar, the changes in electrolyte excretion do not account for the PGE<sub>2</sub>-induced increase in renin secretion. PGE<sub>2</sub> has been shown to increase renin release *in vitro* (15). Although Weber *et al.* did not report PGE<sub>2</sub> to increase renin release, arachidonic acid, PGE<sub>2</sub>, and endoperoxide I and II all increased renin release *in vitro* (16). In the present experiments, both the hemodynamic and tubular responses produced by PGE<sub>2</sub> appear to be similar to those elicited by bradykinin, eleodisin, and acetylcholine *in vivo*. Thus, PGE<sub>2</sub> may increase renin secretion by a direct ac-

TABLE I. EFFECT OF ACETYLCHOLINE, BRADYKININ, AND ELEDOISIN ON SODIUM AND POTASSIUM EXCRETION.

Treatment	Na excretion <sup>a</sup>	K excretion <sup>a</sup>
Acetylcholine	301.2 ± 135.2	26.2 ± 12.3
Bradykinin	385.7 ± 212.1	75.9 ± 30.6
Eleodisin	121.4 ± 36.6	22.5 ± 12.3
Control	478.0 ± 231.3	118.8 ± 51.6

Values expressed as percent increase.

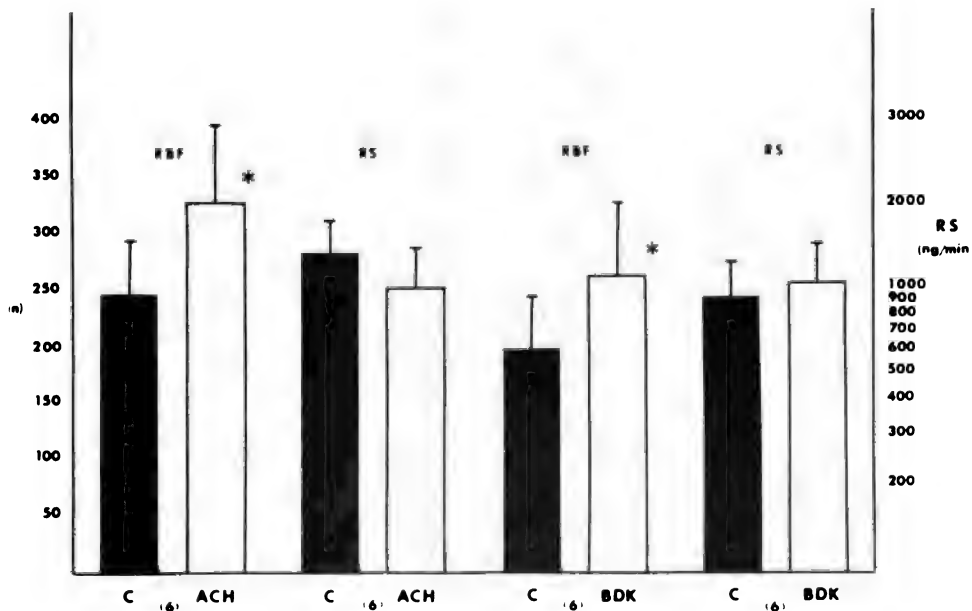


FIGURE 2. Effect of bradykinin (Bdk) and acetylcholine (Ach) on renal blood flow (RBF) and renin secretion (RS). Control. Values are expressed as means ± SEM. n = 6. \*p < 0.05.

tion on the vascular juxtaglomerular cells or a component of the juxtaglomerular apparatus.

**Summary.** Intrarenal infusion of acetylcholine, bradykinin, eledoisin, and PGE<sub>2</sub> increased renal blood flow to a similar degree. Sodium and potassium excretion were similarly affected by each vasodilator. Renin secretion increased following PGE<sub>2</sub> but was unaffected by acetylcholine, bradykinin, or eledoisin. It is suggested that PGE<sub>2</sub> increases renin secretion by a direct effect on the juxtaglomerular apparatus.

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# 9- $\beta$ -Arabinofuranosyladenine Inhibition of Chemically Induced Rat Embryo Cell Transformation (40326)

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Antileukemic chemotherapeutic drug, 9- $\beta$ -arabinofuranosylcytosine (ara-C) was previously shown to be an *in vitro* transforming agent (1) for an established line of Fischer rat embryo cells, which had previously been shown to be an accurate and sensitive indicator of chemicals having carcinogenic properties (2, 3). We were interested in using this system to examine the transforming activity of 9- $\beta$ -D-arabinofuranosyladenine (ara-A), an analog of ara-C which is also used clinically as a cancer chemotherapeutic and antiviral agent in humans (4, 5). The antitumor and antiviral activities of both ara-A and ara-C appear to be derived from inhibition of DNA synthesis (6-8). We were interested that unlike ara-C, ara-A is not a transforming agent for Fischer rat embryo cells (7, 106). Further, nontoxic levels of ara-A protect the cells from transformation in the presence of the known polycyclic hydrocarbon carcinogen, 3-methylcholanthrene (MCA). *Materials and methods.* (A) *Toxicity testing.* Transformation was determined by plating efficiency relative to a control. A control was used to determine the effect of ara-A. Five hundred cells (F1706) were plated in 5 ml of the complete growth medium (minimum essential medium in salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units of penicillin, 100  $\mu$ g of streptomycin/ml) were added to 60-mm plastic cell culture dish (Lux). The dishes were incubated overnight at 37°C in humidified 5% CO<sub>2</sub>-in-air incubator. The following morning the medium was decanted and replaced with a fresh medium containing concentrations of ara-A which had been diluted into the growth medium. Five days later the dishes were fixed and stained (methanolic carbol fuchsin) and macroscopic colonies were counted.

*Transformation assay.* In two separate experiments run concurrently by two different investigators, F1706 D95 cells were inoc-

ulated into 75-cm<sup>2</sup> plastic cell culture flasks (Lux) at a concentration of 10,000 cells/ml and 14 ml per flask. On Days 2 and 5, cultures were refed with either growth medium alone or growth medium containing either 0.01 or 1.0  $\mu$ g/ml ara-A. On Day 6, the cells from each group were transferred to fresh cultures in their respective media at a concentration of 1000 cells/ml and 10 ml per flask. The next day, 10 ml of growth medium was added to one-half the cultures from each group (without decanting the old media), and 10 ml of medium containing 0.4  $\mu$ g/ml of MCA to the other half. MCA was diluted in acetone to 1000  $\mu$ g/ml and was further diluted in the growth medium. After an additional 2 days of incubation, the medium was decanted, and the cultures were washed with growth medium and refed with growth medium still supplemented with ara-A, but no longer containing the MCA. Three days later the cultures were again refed, but now with a growth medium void also of ara-A. The next day, new cultures were initiated at 500 cells/ml. This treatment schedule resulted in the following duplicate sets of cultures: media only (negative control), 0.2  $\mu$ g/ml MCA (positive control), 0.01  $\mu$ g/ml ara-A, 1.0  $\mu$ g/ml ara-A, 0.01  $\mu$ g/ml ara-A plus 0.2  $\mu$ g/ml MCA, and 1.0  $\mu$ g/ml ara-A plus 0.2  $\mu$ g/ml MCA. At each subculture following the initial treatment, one set of flasks was set aside to be held without subdivision (holding series), and the other set subdivided 1:2 weekly to provide two new sets of cultures, one for the holding series and one for subdivision. Transformation was determined by the appearance of foci of cells lacking contact inhibition and orientation and by the formation of macroscopic colonies in semisolid agar (9). Tumorigenicity was determined by subcutaneous inoculation of  $5 \times 10^5$  cells into newborn Fischer rats (F344/f Mai).

*Results.* We routinely test each compound for oncogenic potential at approximately the



LD30 (concentration reducing the relative plating efficiency by approximately 30%) and at the highest concentration resulting in no reduction in relative plating efficiency (MNTD or maximum nontoxic dose). For ara-A these levels were 1.0 and 0.01  $\mu\text{g/ml}$ , respectively (Table I).

At neither level did ara-A, itself, induce cell transformation of F1706 cells. However, as expected, cells treated with 0.2  $\mu\text{g/ml}$  MCA were phenotypically transformed by the third vertical subculture (D + 3), and when tested

at D + 6 produced macroscopic colonies in semisolid agar. When tested at D + 3, all cultures were negative for growth in agar. Cultures treated with MCA in the presence of either level of ara-A were still phenotypically normal at the termination of the experiment 8 subcultures after treatment and failed to grow in semisolid agar when tested at D + 3 and D + 6. When inoculated into the newborn Fischer rats at D + 8, the cultures treated with MCA alone were tumorigenic. The first tumor was found 52 days postinoculation and by the 82nd day, 11 of the 14 rats were positive. In contrast, a total of 45 rats inoculated with cells from cultures treated 8 subcultures earlier with either ara-A or MCA in the presence of ara-A were still tumor free when the experiment was terminated 94 days postinoculation (Table II).

**Discussion.** Many drugs used in cancer chemotherapy are transforming agents (1, 10, 11), mutagens (12), and oncogens (13, 14). One such agent, ara-C, had previously been found to induce transformation in mass cultures of secondary hamster embryo cells (15). This observation was later confirmed using a quantitative hamster transformation system, as well as the F1706 cells used in the present study (1). Subsequently, it was demonstrated,

TABLE I. TOXICITY OF ara-A<sup>a</sup> AS DETERMINED BY REDUCTION IN PLATING EFFICIENCY OF F1706 D95<sup>b</sup>.

Concentration ( $\mu\text{g/ml}$ )	Relative plating efficiency <sup>c</sup> (%)
100	21
10	45
1.0	73
0.1	87
0.01	95
0.001	100

<sup>a</sup> 9- $\beta$ -D-Arabinofuranosyladenine.

<sup>b</sup> A serial line of Fischer rat embryo cells in its 95th population doubling.

<sup>c</sup> The percentage of cells giving rise to macroscopic colonies, relative to the media only control, in which the absolute plating efficiency was arbitrarily set at 100%. The absolute plating efficiency of the control was 20% (108 colonies out of 500 cells plated).

TABLE II. MCA<sup>a</sup>-INDUCED TRANSFORMATION OF F1706<sup>b</sup> AND PROTECTION FROM TRANSFORMATION BY ara-A.

Treatment (per ml)	Morphological transformation <sup>c</sup>	Growth in agar (D6) <sup>d</sup>	Tumor results, <sup>e</sup> No. positive/No. inoculated (days to 1st tumor-days to last tumor)
Media control	— (+8)	—	ND <sup>e</sup>
Media control	— (+8)	—	0/5
0.2 $\mu\text{g/ml}$ MCA	+ (+3)	+	11/12 (56-82)
0.2 $\mu\text{g/ml}$ MCA	+ (+3)	+	0/2 <sup>f</sup>
1.0 $\mu\text{g}$ ara-A	— (+8)	—	0/9
1.0 $\mu\text{g}$ ara-A	— (+8)	—	0/13
0.01 $\mu\text{g}$ ara-A	— (+8)	—	ND
0.01 $\mu\text{g}$ ara-A	— (+8)	—	ND
1.0 $\mu\text{g}$ ara-A + 0.2 $\mu\text{g}$ MCA	— (+8)	—	0/10
1.0 $\mu\text{g}$ ara-A + 0.2 $\mu\text{g}$ MCA	— (+8)	—	0/13
0.01 $\mu\text{g}$ ara-A + 0.2 $\mu\text{g}$ MCA	— (+8)	—	ND
0.01 $\mu\text{g}$ ara-A + 0.2 $\mu\text{g}$ MCA	— (+8)	—	ND

<sup>a</sup> 3-Methylcholanthrene.

<sup>b</sup> A serial line of Fischer rat embryo cells.

<sup>c</sup> Newborn Fischer rats inoculated with  $5 \times 10^5$  cells (0.05 ml) from D + 8. Rats without tumors were held 94 days and then sacrificed.

<sup>d</sup> Triplicate agar dishes were each inoculated with 50,000 cells from cultures at D + 6 (6 population doublings after removal of the MCA), held 4 weeks at 37° in a humidified 5% CO<sub>2</sub> incubator, and screened for the appearance of macroscopic colonies.

<sup>e</sup> Not done.

<sup>f</sup> Twelve rats inoculated, 10 killed by mother.

using the C<sub>3</sub>H/10T1/2 mouse embryo cells (16), that oncogenic transformation took place maximally in the S phase of the cell cycle (17). We know from double-blind studies that 90% of the chemicals which transform these cells are also oncogenic for mice and rats (2). Since it is possible that tumor induction in the rodent may be relevant to tumor induction in man, it seems wise to avoid where possible the use of chemotherapeutic agents which transform rodent cells. Ara-C is a transforming agent. Ara-A did not transform the F1706 rat cells, and at nontoxic doses protected the cells from transformation induced by the potent carcinogen, MCA.

We have previously used this *in vitro* system (F1706) to show that several antiviral antibiotics, i.e., streptonigrin (18), cordycepin (19), and geldanamycin (20), could protect the cells from chemically induced transformation. We suggested that this protection was due to the ability of the antibiotic to inhibit endogenous oncornavirus expression, since each drug also inhibited the "turn-on" of endogenous virus by halogenated pyrimidines. This explanation, however, is not applicable to ara-A protection of MCA-induced cell transformation, since ara-A did not inhibit transient virus induction by halogenated pyrimidines under similar conditions.

These studies suggest that *in vitro* cell transformation assays may have value, not only as a prescreen for potentially oncogenic chemicals, but also for compounds having anticancer properties.

**Summary.** The cancer chemotherapeutic and antiviral agent 9- $\beta$ -D-arabinofuranosyladenine (ara-A) was examined for potential oncogenicity, using a serial line of Fischer rat embryo cells, which was previously shown to be a sensitive and accurate indicator of chemicals carcinogenic for rodents. We report here that at the concentrations tested, ara-A was not a transforming agent. Further, ara-A protected the cells from transformation induced by the known carcinogen, 3-methylcholanthrene.

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# Prolactin Receptors in Mouse Liver: Species Differences in Response to Estrogenic Stimulation<sup>1</sup> (40327)

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Specific prolactin (PRL) receptors have been demonstrated in the liver of many species, including rats and mice (1-3). Ovariectomy (OVX) decreased and estrogen replacement increased PRL binding sites. The inductive effects of estrogen on PRL binding in the liver was dose related in OVX rats, and anti-estrogens reduced PRL receptors in the liver of female rats (4). One mechanism whereby estrogen induced PRL receptors is by stimulation of pituitary PRL release, resulting in induction of hepatic PRL binding sites in the liver (5, 6). However, since very low doses of estrogen increased PRL binding in the liver without altering serum PRL levels (4), and since the PRL-inhibiting ergot drug CB-154 did not decrease the estrogen-induced increase in hepatic PRL binding sites (4), it is possible that estrogen may act directly on the liver to increase PRL receptors.

All of the above studies were performed in rats. To determine whether the estrogen effect on PRL receptors was observable in other species, we examined the effects of estrogen on PRL receptors in the liver of mice. The results indicate that estrogen inhibits induction of PRL receptors in the liver of female mice, in contrast to its stimulation of PRL receptors in the liver of male and female rats.

**Materials and methods.** Adult male and female Swiss-Webster mice were obtained from Spartan Research Animals, Haslett, Michigan. Mice were housed in a temperature-controlled ( $25 \pm 1^\circ$ ) and artificially illuminated room (lights on from 0500 to 1900

hr daily) and received food and water *ad libitum*.

**Experiment 1.** Female mice were OVX on Day 1 and were injected sc daily with 2  $\mu$ g of estradiol benzoate (EB) in 50  $\mu$ l of corn oil on Days 8 through 14. On the 15th day all OVX were killed together with a group of intact females which were similarly injected daily on Days 8 through 14 with vehicle alone.

**Experiment 2.** Female mice, OVX 14 days prior to estrogen treatment, were given daily sc injections of either 1, 10, 20, or 50  $\mu$ g of EB in 50  $\mu$ l of corn oil. Mice were then killed after 12 days of treatment, together with groups of intact and OVX controls which were injected with vehicle alone. Additional treatment groups given daily injections of 20  $\mu$ g of EB were killed after 6 or 9 days of treatment.

**Experiment 3.** Male mice were given a single 2- $\mu$ g EB sc injection in 50  $\mu$ l of corn oil and killed 7 days later. Controls were injected with vehicle alone.

At the end of each experiment the mice were anesthetized with ether and decapitated, and the blood obtained from the cervical wound was allowed to clot at  $4^\circ$ . The serum was separated by centrifugation and stored at  $-20^\circ$  for later serum PRL measurements. Livers were removed and a microsomal membrane fraction was obtained by differential centrifugation as described previously (1). PRL was iodinated by a lactoperoxidase method (1) and the binding of [ $^{125}$ I]iodo-PRL to liver membranes was determined. Incubations with membrane protein and [ $^{125}$ I]iodo-PRL were performed at  $4^\circ$  for 60 hr, in the presence of excess (1  $\mu$ g) unlabeled PRL and in its absence. Livers from female mice were assayed for PRL binding, using 300  $\mu$ g of membrane protein per tube, whereas for male livers 1000  $\mu$ g per tube was used. Specific binding refers to the difference in radioactiv-

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bound to membranes after incubations with and without unlabeled PRL, and for each of representation is expressed as a percentage of the total counts added. PRL binding to liver membranes from mice has been shown to be both time and temperature dependent, and specific for lactogenic hormones. Mouse PRL was measured by a double antibody radioimmunoassay using the materials and methods of Sinha *et al.* (7). The biological potency of the mouse PRL standard was 25.0 IU/mg. The data in Expts 1 and 2 were treated by an analysis of variance of unequal sample size, followed by a Student-Neuman-Kuels test for comparison of means among groups. Student's *t* test was used to determine significance in Expt 3. *P* 0.05 was considered to be significant.

**Results.** Figure 1 shows that OVX significantly increased (*P* < 0.01) [<sup>125</sup>I]iodo-PRL binding to mouse liver membranes and that this enhanced binding could be decreased to intact control values by estrogen replacement. When this experiment was repeated (Fig. 2) with various doses of EB and longer treatment times, similar results were obtained. OVX increased (*P* < 0.05) specific [<sup>125</sup>I]iodo-PRL binding from 14.48 ± 0.85% in the intact controls to 19.93 ± 0.60%. Replacement by estradiol 1 and 10 µg of EB for 12 days reduced PRL binding to 11.84 ± 0.53 and 9.0 ± 0.81%, respectively, which were not significantly different from intact control val-

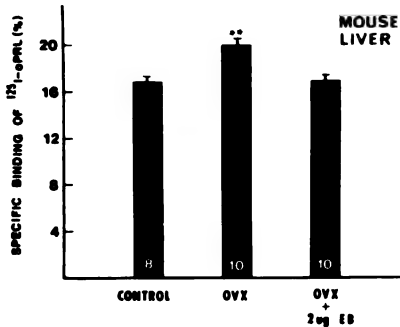


FIG. 1. Effects of OVX and OVX with EB replacement on specific [<sup>125</sup>I]iodo-PRL binding to liver membrane preparations from female mice. For each tissue sample, incubations were performed in triplicate at 4° for 60 hr, using 300 µg of membrane protein per tube. The amount of [<sup>125</sup>I]iodo-PRL per tube was 1.0 × 10<sup>5</sup> cpm. The line above each bar represents 1 SEM, and the numbers in white indicate the number of observations per group. \*\**P* < 0.01 when compared to intact controls.

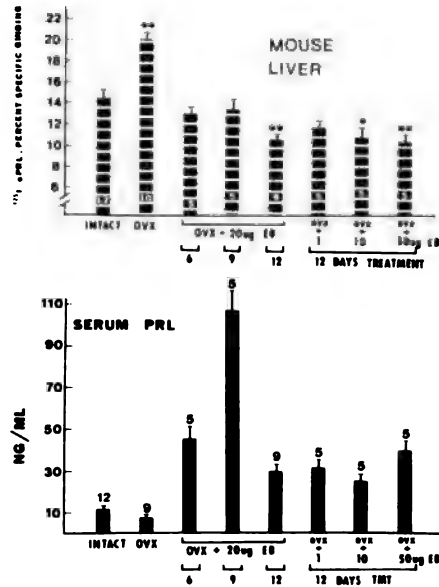


FIG. 2. Serum PRL levels and [<sup>125</sup>I]iodo-PRL binding to liver membranes from intact and OVX mice and OVX mice given daily injections of different doses of EB. For each tissue sample, incubations were performed in triplicate at 4° for 60 hr, using 300 µg of membrane protein per tube. The amount of [<sup>125</sup>I]iodo-PRL per tube was 1.0 × 10<sup>5</sup> cpm. The line above each bar represents 1 SEM, and the numbers in white indicate the number of observations for each group. \**P* < 0.05 as compared to intact controls. \*\**P* < 0.01 as compared to intact controls.

ues, whereas 20 and 50 µg of EB significantly reduced binding to below intact levels. Serum PRL was reduced from 12.0 ± 1.5 ng/ml (intact controls) to 8.09 ± 2.0 ng/ml in the OVX rats. All estrogen-treated groups had serum PRL values significantly higher than those in intact controls.

Figure 3 demonstrates the effects of a single injection of 2 µg of EB on specific PRL binding sites in liver membranes obtained from male mice. PRL binding increased (*P* < 0.01) from 22.61 ± 1.16 to 33.72 ± 1.29% at 7 days postinjection. Since PRL binding sites on male liver membranes were measured using 1000 µg of membrane protein rather than 300 µg of membrane protein (as used in quantitating PRL receptors in the liver of females), specific binding is higher in the livers of females than in the livers of males when compared on a milligram of protein basis. This is in agreement with the data of Posner (3).

**Discussion.** The presence of specific PRL

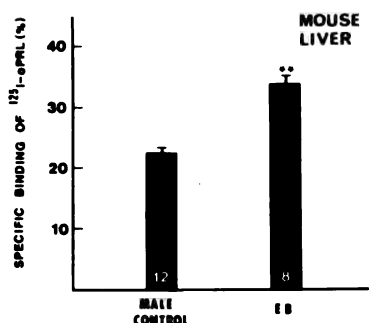


FIG. 3. Specific binding of [<sup>125</sup>I]iodo-PRL to liver membranes from male mice 7 days after a single injection of EB. For each tissue sample, incubations were performed in triplicate at 4° for 60 hr, using 1000 µg of membrane protein per tube. The amount of [<sup>125</sup>I]iodo-PRL per tube was  $1.0 \times 10^5$  cpm. The line above each bar represents 1 SEM, and the number in white indicates the number of observations for each group. \*\**P* < 0.01 as compared to male controls.

receptors in liver membranes of female mice agrees with the findings of other investigators (3, 8). However, our results indicate that OVX results in an increase of hepatic PRL receptors in female mice, whereas estrogen treatment over a large dose range reduced PRL binding to intact or below intact values. These data in female mice represent a striking contrast to the effects of OVX and estrogen replacement on PRL receptors in liver of female and male rats.

In female rats the effects of estrogen on increasing hepatic PRL receptors was convincingly demonstrated to be mediated through stimulation of pituitary PRL release (5, 9). However, other data suggest a direct effect of estrogen on the liver to modulate PRL binding sites (4). In the present study, all doses of estrogen significantly increased serum PRL levels in female mice. The increase in PRL, however, is not believed to have altered hepatic PRL receptors since other investigators have reported that neither the high levels of endogenous PRL during pregnancy, nor exogenous PRL injections to female mice, influenced PRL binding sites in the liver (3, 8). Therefore, a direct effect on the liver appears likely, although an indirect effect of estrogen cannot be excluded.

In male mice a single injection of 2 µg of EB was able to significantly increase PRL binding sites in the liver. Since estradiol valerate has been reported to stimulate PRL

binding sites in the liver of male rats (5) apparent that both male rats and male mice respond similarly to the stimulatory action of estrogen on hepatic PRL receptors. This contrast to the opposite effects of estrogen on hepatic PRL binding sites of female rats and mice.

Although the physiological significance of these results is not known at this time, it has been shown to have numerous effects on liver function of various species. Thus, estradiol was reported to regulate free fatty acid synthesis in dog (10) and rat (11) livers, stimulate hepatic RNA synthesis in dwarf mice, modulate ornithine decarboxylase activity in the liver of rats (13), and increase somatomedin release from rat livers (14). However, in order for PRL to exert an effect on a target cell, it must first bind to a stereospecific plasma membrane receptor to induce intracellular changes. Consequently, receptor modulation could provide a mechanism for altering the sensitivity of target organs to circulating PRL. Therefore, determining which hormones can alter PRL receptor function and the direction of these changes are important for clarifying the physiological actions of PRL on liver function.

The present data clearly demonstrate an important species difference between female rats and mice in estrogenic control of hepatic PRL receptors and may have several implications. Thus, the use of the rat as a model for investigating factors modulating PRL receptors in the liver cannot be considered valid for other species. Moreover, the function of PRL on liver function may be different between males and females of even the same species, since control of PRL receptors in the liver of male and female mice are different. Our data indicate that estrogen inhibits PRL binding sites in the female, whereas in the male, binding is stimulated. Thus, the response of hepatic PRL receptors to estrogen is both species and sex dependent. The mechanisms of action by which these effects are mediated remain to be clarified. The differential findings in these two species need to be considered when designing and interpreting studies on the effects of PRL on liver function.

**Summary.** Serum PRL and hepatic PRL receptors were measured in intact and

and OVX mice given several doses of OVX significantly increased PRL binding in the liver of female mice, and EB reduced receptors to intact or below intact levels. It was concluded that estrogen decreases receptors in the liver of female mice. This is a striking contrast to the stimulatory effect of estrogen on hepatic PRL receptors in male and female rats. EB elevated serum PRL in OVX mice, but since other investigators reported that PRL does not alter hepatic PRL receptors in female mice, it appears that estrogen reduced PRL binding by a direct effect on the liver. However, a direct effect cannot be excluded. In male mice, estrogen increased PRL receptors in the liver as in male rats.

The present data demonstrate important sex differences between female rats and female mice in estrogenic control of hepatic PRL receptors. Moreover, the inhibitory effect of estrogen in female mice, and its stimulatory action in male mice, suggest that the response of hepatic PRL receptors to estrogen may be sex dependent in different species. The mechanisms of action by which these effects are mediated remain to be clarified.

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# Hemopoiesis in Diffusion Chambers in Strontium-89 Marrow-Ablated Mice<sup>1</sup> (40328)

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Hemopoiesis can be evaluated by studying the proliferation of hemopoietic cells in diffusion chambers (DCs) implanted into the peritoneal cavities of animals. During the initial several hours after seeding and implantation, the number of cells recoverable from the inoculum declines by 40 to 60% (1).

The predominant hemopoietic precursor cell responsible for the enlarging hemopoietic cell population in DCs appears to be a granulocyte/macrophage committed precursor (2, 3). Multipotent hemopoietic stem cells (CFU-S), however, also have been shown to play a role in DC hemopoiesis (2, 4-7) and the number of these cells reaches its peak in DC cultures before the total number of hemopoietic elements reaches its maximum (8, 9).

If the hemopoietic cell inoculum consists of steady state cells, such as cells from marrows of normal mice, proliferation in DCs begins after a lag of about 18 hr (1). A number of investigators have found that in DCs the number of cells harvested, at least from Days 4 to 7 after implantation, is related linearly to the number of cells in the inoculum, suggesting that there is little or no significant cell-cell interaction (10-12). Niskanen and his colleagues (13, 14), on the other hand, found that as the numbers of cells seeded in DCs were increased, the growth of both differentiated granulocytes as well as CFU-S was inhibited. In addition, proliferation of cells in DCs is modified substantially by pretreatment of the host animals with agents such as irradiation (8, 10, 15, 16) or cytotoxic drugs (2, 4, 10, 13, 15, 17), both of which perturb the hemopoietic state of host animals.

Elevated levels of colony-stimulating activity (CSA), i.e., glycoproteins required for the growth of granulocyte/macrophage precursors *in vitro*, have been found in the serum of animals treated with whole-body irradiation (13, 16, 18-20). In whole-body-irradiated animals, hemopoiesis in DCs has been found to parallel the increase in serum CSA levels (15, 16); this relationship is expected, as the DC technique primarily assesses granulopoiesis.

The bone-seeking radionuclide, <sup>89</sup>Sr, can be used to ablate marrow hemopoiesis selectively (21-24). By 10 days after <sup>89</sup>Sr injection (4  $\mu$ Ci/g body wt) the marrows of mice are aplastic and contain less than 2% of the normal number of CFU-S (24). The spleens of <sup>89</sup>Sr-treated mice support marked compensatory-hemopoiesis and these mice develop only a mild anemia but a more severe leukopenia; with the passage of time, hemopoiesis is gradually restored in the marrows of these mice (21, 24, 25). In a previous study, we did not detect an elevation in serum CSA levels in <sup>89</sup>Sr-treated mice (26).

In an attempt to evaluate the presence of a humoral stimulus for hemopoiesis in <sup>89</sup>Sr-treated mice, at various times after <sup>89</sup>Sr treatment, we implanted into such mice DCs containing  $1 \times 10^6$  marrow cells from normal mice. We evaluated the total number of cells, proportions of the various cellular elements, and the number of CFU-S in the DCs 72 hr after implantation.

**Materials and methods.** Pathogen-free female CAF<sub>1</sub> (Balb/c  $\times$  A/He) mice (Cumberland View Farms, Clinton, Tenn.), 14 to 16 weeks old, were housed in cages with disposable plastic bottoms; a maximum of 10 mice were housed per cage. The mice were permitted food and acidified (pH 3.2) water *ad libitum*. On Day 0 the mice were given ip injections of <sup>89</sup>SrCl<sub>2</sub> (Oak Ridge National Laboratories, Oak Ridge, Tenn.), 4  $\mu$ Ci/g body wt, in 0.25 ml of a solution buffered to pH 5 to 6; control mice were injected with a comparable amount of cold <sup>89</sup>SrCl<sub>2</sub>. On days

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3, and 39 after the Sr injections, DCs planted into the peritoneal cavities of anesthetized (sodium pentobarbital) mice. 12 hr later the DCs were removed. Prior to use, blood was obtained from each mouse by bleeding it from the lateral tail vein into heparinized capillary tubes. For a microhematocrit determination, total white cell count (by hemacytometer), and 100-cell differential count were performed on the blood from each mouse. For the study, five radio- $^{89}\text{Sr}$ -treated and five  $^{86}\text{Sr}$ -treated mice were studied. Each mouse was implanted with two DCs, one into the right side and the other into the left side of the peritoneal cavity; the chambers were labeled for identification prior to implanta-

tion. The DCs were constructed by gluing deionized polycarbonate membranes (Nuclepore Corp., Berkeley, Calif.) which had  $0.22\text{-}\mu\text{m}$  pores, between two sides of plastic rings (Millipore, Bedford, Mass.) with Millipore MF 1000 adhesive. The DCs were tested for leaks by filling with air under water and then sterilizing at  $70^\circ\text{C}$  dry heat for 16 hr. They were filled with  $1 \times 10^6$  marrow cells pooled from donors of three CAF<sub>1</sub> mice; the cells were suspended in 0.1 ml of Hanks' balanced salt solution (HBSS). The holes used to fill the DCs were occluded with plugs of dental wax. Prior to implantation, the DCs were immersed in a solution of penicillin and streptomycin. After 72 hr in the mice, the chambers were removed and placed into a solution containing 0.5% grade B Pronase (Calbiochem, San Diego, Calif.) and 5% Ficoll (Lithium Chloride, Lab Products, Kensington, Md.) in which they remained for 90 min at room temperature; they were agitated continuously. The wax plugs were removed and the contents of the DCs were removed by aspiration through the filling hole by means of a 25-gauge needle attached to a tuberculin syringe. The chambers were washed thrice with HBSS; the last wash was performed for the removal of one of the Nuclepore membranes. At each time studied, the contents of the five chambers which were implanted into the right sides of the mice were pooled to form one suspension of pooled cells and those from the five chambers from the left sides another. These two suspensions

of pooled cells were counted and assayed separately. Cytospin centrifuge (Shandon Southern Instruments, Inc., Sewickley, Penn.) slides were prepared from each suspension of pooled cells and a 400-cell differential count was performed on each of the suspensions. The criteria of Benestad (27) were used to classify proliferative and nonproliferative granulocytic elements. Duplicate nucleated cell counts were performed on each cellular suspension by means of a hemacytometer.

The CFU-S content of each cellular suspension was assayed by the surface spleen colony technique of Till and McCullough (28). The pooled cellular suspensions were diluted so that the equivalent of  $\frac{1}{100}$ th or  $\frac{1}{1000}$ th of the contents of a single chamber was contained in 0.5 ml of HBSS which was then injected into a lateral tail vein of an assay mouse which had been exposed to 900 rad of whole-body irradiation provided by a  $^{137}\text{Cs}$  source (Gamma Cell 40, Atomic Energy of Canada, Ltd., Ottawa, Canada) within the previous 3 hr. We used 12 to 15 mice to assay each suspension of cells.

The results of the studies performed on the chambers implanted into the right and left sides of the mice were evaluated separately; as the results from the two groups were virtually identical we will report only the pooled data. We had control studies at each time interval and report the results of the cell counts and CFU-S assays individually. The differential counts performed on the contents of the chambers implanted into the control mice were very similar at the four times evaluated; this is to be expected, as the control mice, injected with  $^{86}\text{SrCl}_2$  were "normal" animals at all times. To simplify the reporting of differential counts of the DC cells, we have reported the differential counts from the cells implanted into the normal mice as means  $\pm$  SE obtained from all the time intervals studied; the results from the experimental mice are reported separately for each time.

Student's *t* test was used to evaluate the statistical differences between the results obtained from the  $^{89}\text{Sr}$  and  $^{86}\text{Sr}$  groups. As there are only two values (obtained from the right and left chamber suspensions) for the total numbers of cells per chamber and for the differential counts of the cells for each group at each time studied, we did not analyze these



statistically (Fig. 1C; Table I).

**Results.** The  $^{89}\text{Sr}$ -treated host mice were significantly anemic only during the period in which the second group of chambers were implanted, i.e., 10 to 13 days after  $^{89}\text{Sr}$  injection (Fig. 1A), but these mice were granulocytopenic at all times studied (Fig. 1B).

The total number of nucleated cells harvested from the DCs implanted into the  $^{89}\text{Sr}$ -treated mice was greater than that harvested from the DCs housed in the control mice at all times studied (Fig. 1C); the largest differences occurred at the 10- to 13-day and 18- to 21-day time periods when the ratios between the cell contents of the DCs from the  $^{89}\text{Sr}$  and those from the  $^{89}\text{Sr}$  control mice were 1.8 and 2.4, respectively (Fig. 1C). In addition, at the first three times studied, the numbers of cells harvested from the chamber housed in the  $^{89}\text{Sr}$ -treated mice exceeded the numbers ( $1 \times 10^6$  cells) in the original inoculum.

In general, the proportion of the various cellular elements in the DCs of the  $^{89}\text{Sr}$  and

$^{89}\text{Sr}$  mice were quite similar (Table I). There was, however, a slight increase in the proportion of blasts in the DCs from the  $^{89}\text{Sr}$  mice during the first three times studied (Table I). In addition, in the 10- to 13-day DCs from the  $^{89}\text{Sr}$ -treated mice there was a modest increase in the proportion of nucleated red blood cells (Table I); this was the only time during which the  $^{89}\text{Sr}$ -treated mice were significantly anemic (Fig. 1A).

The inoculum contained about 340 CFU-S. The numbers of CFU-S harvested from the DCs housed in the  $^{89}\text{Sr}$ -treated mice were significantly greater than those from the DCs housed in the  $^{89}\text{Sr}$  control mice. The greatest difference between the numbers of CFU-S in the two groups occurred in those chambers implanted during the second (10–13 days) and third (18–21 days) intervals studied (Fig. 1D); these were the same times during which the largest differences were found in total numbers of nucleated cells per chamber. The second-interval-chambers, implanted 10 days after  $^{89}\text{Sr}$  injection, contained more than

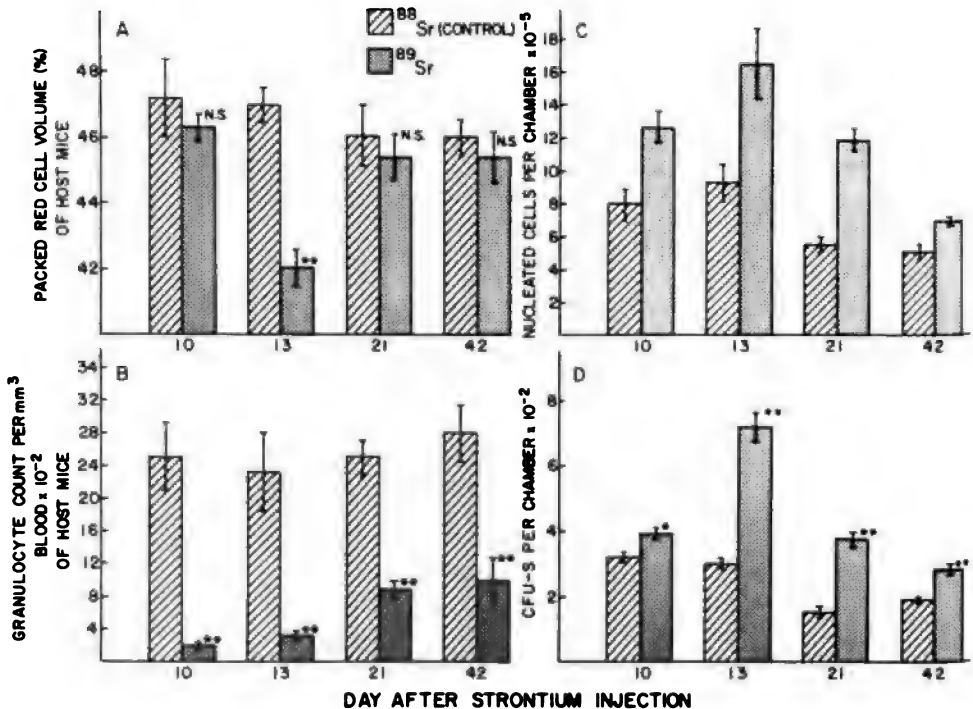


FIG. 1. (A) Packed red cell volume (as a percentage) and (B) granulocyte counts per cubic millimeter of blood from diffusion chamber (DC) host mice; and (C) nucleated cell counts and (D) numbers of CFU-S of DCs. All chambers were in mice for 72 hr. Days indicated are numbers of days after Sr injections which also were days on which blood counts were performed and DCs harvested. Means  $\pm$  SE. N.S., not significant; \*p < 0.01; \*\*p < 0.001.

vice as many CFU-S as did the inoculum.

**Discussion.** The larger number of CFU-S in DCs cultivated in  $^{89}\text{Sr}$ -treated mice as compared to that in DCs from control mice suggests that in  $^{89}\text{Sr}$ -treated mice there is a humoral mechanism(s) which effects either more rapid proliferation of CFU-S or a shortening of the preproliferative lag period or both. The early (by 72 hr) substantial increase in the number of differentiated blood elements in DCs from  $^{89}\text{Sr}$ -treated mice suggests that there also is a stimulus for the proliferation of committed precursor cells.

In Table II we have summarized studies

from the literature on the growth of cells in DCs implanted into hemopoietically stressed mice in which both cell numbers and CFU-S were studied early after DC implantation. The increase in DC contents above that in the inoculum in our  $^{89}\text{Sr}$ -treated mice occurred as early (Day 3) as a similar increase in 800-rad whole-body-irradiated (WBI) mice (8) (Table II); on Day 3, the magnitude of the increase in DCs from  $^{89}\text{Sr}$ -treated mice may even have been slightly greater than that in DCs from 800-rad WBI mice (8). Moreover, if cell density does influence the growth rate of cells in DCs, the increase noted in our

TABLE I. DIFFERENTIAL COUNTS, AS PERCENTAGES, OF CELLS FROM MICE USED TO INOCULATE DIFFUSION CHAMBERS (DCs) AND OF CELLS HARVESTED FROM DCs CULTIVATED IN  $^{89}\text{Sr}$ - OR  $^{89}\text{Sr}$ -TREATED MICE.

	Blasts	Proliferative granulocytes	Nonproliferative granulocytes	Monocytes and macrophages	Red cell precursors	Lymphocytes	Other <sup>a</sup>
Inoculum <sup>b</sup>	2.1 $\pm$ 0.3	11.1 $\pm$ 1.3	30.3 $\pm$ 3.0	0.6 $\pm$ 0.2	30.0 $\pm$ 1.4	20.6 $\pm$ 0.6	5.3 $\pm$ 1.2
$^{89}\text{Sr}$	1.0 $\pm$ 0.7	19.6 $\pm$ 3.3	42.0 $\pm$ 2.1	26.1 $\pm$ 1.6	1.0 $\pm$ 0.9	7.2 $\pm$ 1.7	3.1 $\pm$ 1.8
$^{89}\text{Sr}$							
Day <sup>c</sup>							
10	2.3	18.7	39.2	33.8	0.5	4.7	0.8
13	2.2	19.5	43.7	25.3	5.5 <sup>d</sup>	2.5	1.3
21	2.0	18.8	48.2	23.8	1.0	4.0	2.2
42	1.5	19.0	38.5	27.5	1.3	8.3	3.9

<sup>a</sup> This category includes: basophils, eosinophils, plasma cells, megakaryocytes, and cells in mitoses.

<sup>b</sup> For each interval, cells pooled from the femurs of three normal CAF<sub>1</sub> mice were used to inoculate the DCs.

<sup>c</sup> Data for DCs cultivated in  $^{89}\text{Sr}$ -treated mice are values pooled from all four times studied; means  $\pm$  SE.

<sup>d</sup> Data for DCs cultivated in  $^{89}\text{Sr}$  mice are averages of data obtained from two groups (right and left) of chambers at each time.

<sup>e</sup> Day after injection of  $^{89}\text{Sr}$ ; this was the day on which chambers were harvested. All chambers were in mice for 2 hr.

<sup>f</sup> There were more red cell precursors in DCs implanted into  $^{89}\text{Sr}$ -treated mice on Day 10 and harvested on Day 3; Day 13 was the only time at which  $^{89}\text{Sr}$  mice were substantially anemic (Fig. 1A).

TABLE II. REVIEW FROM THE LITERATURE OF STUDIES IN WHICH BOTH NUMBERS OF CELLS AND CFU-S WERE ASSAYED IN DCs IMPLANTED INTO HEMOPOIETICALLY STRESSED MICE: (A) THE FIRST DAY AFTER DC IMPLANTATION ON WHICH THE CELL POPULATION (TOTAL AND CFU-S) EXCEEDED THAT OF THE INOCULUM AND (B) THE MAGNITUDE OF THIS VERY EARLY INCREASE.

Author (method used to stress mouse hemopoiesis) and size of inoculum	Nucleated cells/chamber <sup>a</sup> Day; magnitude of increase over input <sup>b</sup>	CFU-S/chamber Day; magnitude of increase over input <sup>b</sup>
Niskanen <i>et al.</i> (13) (Cyclophosphamide, 350 mg/kg)		
1 $\times$ 10 <sup>5</sup> nucleated cells	5; 2 $\times$	4; 2 $\times$
5 $\times$ 10 <sup>5</sup> nucleated cells	5; 1.6 $\times$	Not done
Shulman and Robinson (9) (500 R WBI <sup>c</sup> )		
1 $\times$ 10 <sup>5</sup> nucleated cells	4; 3.4 $\times$	6; 1.4 $\times$
Boyum <i>et al.</i> (8) (800 R WBI)		
7 $\times$ 10 <sup>4</sup> granulocytes	3; 1.2 $\times$	3; 1.2 $\times$
Adler and Trobaugh (present study) (4 $\mu\text{Ci/g}$ of $^{89}\text{Sr}$ )		
1 $\times$ 10 <sup>5</sup> nucleated cells	3; up to 1.7 $\times$	3; up to 2 $\times$

<sup>a</sup> For the study of Boyum *et al.* (8) data are granulocytes/chamber rather than nucleated cells/chamber.

<sup>b</sup> In some cases the magnitude of increase had to be approximated from data supplied in the publications.

<sup>c</sup> WBI, whole body irradiation

studies becomes even more striking as we seeded the DCs with  $1 \times 10^6$  cells, substantially more cells than were used in the other studies cited in Table II. The contents of the DCs exceeded the input levels earlier in our  $^{89}\text{Sr}$ -treated mice (Day 3) than they did in cyclophosphamide-treated mice (Days 4-5) (13) even though the latter had a lower neutrophil nadir ( $250/\text{mm}^3$  vs  $1/\text{mm}^3$ ). Thus, it seems that DC growth is influenced not only by the degree of neutropenia but also by the modality used to induce it. This confirms the finding of Brevik and Benestad (7) who have noted that irradiation provides a stronger stimulus for DC chamber than does cytoxan treatment; we might add that  $^{89}\text{Sr}$  irradiation may provide even a stronger stimulus than external WBI.

Although we did not assay the committed granulocyte/macrophage precursor cells (CFU-C), this cell is one of the primary cells which proliferates and differentiates in DCs (9, 14, 29, 30). Beran (15) has shown that the increase in mature cells in DCs implanted into hemopoietically stressed mice from the third day onward is not due to variations in survival times of the cells implanted, rather it is related to proliferative characteristics of the cells and Quesenberry *et al.* (14) have shown that granulocyte production correlates well with CFU-C proliferation. Based on this knowledge it seems reasonable to assume that the larger population of differentiated white cell elements in DCs in  $^{89}\text{Sr}$ -treated mice as compared to that in DCs in control mice results from increased CFU-C proliferation in the DCs implanted into the  $^{89}\text{Sr}$ -treated mice. In spite of the augmented granulopoiesis in  $^{89}\text{Sr}$ -treated mice as measured by the DC assay, in a previous study (26) we were not able to detect any elevated levels of CSA in  $^{89}\text{Sr}$ -treated mice. It may be that for the  $^{89}\text{Sr}$  model, the DC technique is more sensitive to CSA than is the *in vitro* assay for CSA. Alternatively, a factor other than CSA may be responsible for the enhanced granulopoiesis in DCs. Although some investigators have found support for the role of CSA in DC growth (15, 16), Rothstein *et al.* (31) have adduced experimental evidence which casts doubt on the role of CSA in DC hemopoiesis. In any event, the studies reported here underscore the importance of employing

multiple experimental systems before using the presence of a humoral factor of hemopoietic stress.

**Summary.** The numbers of pluripotent stem cells (CFU-S) and of the more differentiated granulocyte/macrophage cells in diffusion chambers (DCs) implanted in the peritoneal cavities of radio- $^{89}\text{Sr}$ -irradiated mice are increased as compared to those in DCs implanted into cold-irradiated mice. These findings suggest that there is a systemic humoral response of stimulating hemopoiesis even in marrow aplastic marrows and whose hemopoiesis is localized to their spleens. The magnitude of this response and the promptness with which the response is manifest in DC growth suggests that marrow aplasia induced by irradiation provides a stronger stimulus for proliferation of cells in DCs than does either cyclophosphamide or lethal external whole-body irradiation.

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# Effect of Long-Term Administration of Epinephrine and Propranolol on Serum Calcium, Parathyroid Hormone, and Calcitonin in the Rat (40329)

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Previous short-term *in vitro* and *in vivo* studies have shown the importance of  $\beta$ -adrenergic stimuli in the secretion of parathyroid hormone (PTH) (1-5) and calcitonin (CT) (6-8). In these studies,  $\beta$ -adrenergic agonists, epinephrine and isoproterenol, increased PTH and CT secretion, whereas the  $\beta$ -adrenergic antagonist, propranolol, inhibited the secretion of these two hormones. Subsequent studies have suggested that the effects of isoproterenol in the perfusion system (9) and of epinephrine *in vivo* in the cow (10) may be short-lived, lasting for 40 to 50 min. The present studies therefore evaluated the effects of long-term administration of epinephrine or propranolol on PTH and CT secretion in the rat.

**Materials and methods.** Sprague-Dawley rats weighing 250 to 300 g were divided into three groups.

Group I rats received daily im injection of 1-epinephrine in sesame seed oil (0.3 mg/day for 2 weeks followed by 0.6 mg/day for an additional 3 weeks) ( $n = 5$ ).

Group II rats received *dl*-propranolol<sup>1</sup> (approximately 40 mg/day) for 5 weeks in their drinking water and in addition received daily im injections of sesame seed oil ( $n = 4$ ).

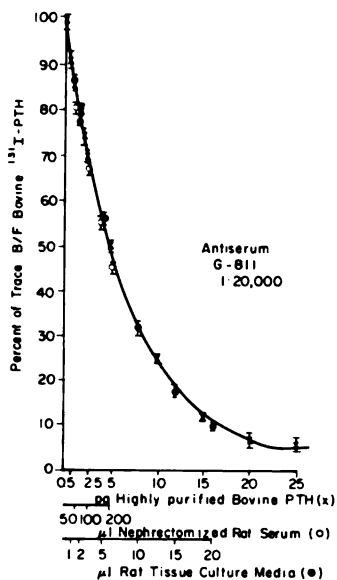
Group III rats served as control and received daily im injections of sesame seed oil ( $n = 6$ ).

All animals were bled via orbital sinus puncture at weekly intervals with bleedings being performed 24 hr after the last injection. Serum was separated within 2 hr of the bleeding and frozen for subsequent analysis for serum PTH, CT, calcium, and total proteins.

<sup>1</sup> *dl*-Propranolol was kindly supplied by the Ayrest Laboratories, New York, New York. Fifty milligrams was dissolved in 50 ml of water and kept in light-proof drinking water bottles. Each rat consumed approximately 40 ml of water daily.

Serum parathyroid hormone was assayed by a slight modification of the previously described method for rat PTH developed in our laboratory (11). The method utilizes an antibody against parathyroid hormone developed in our laboratory. Figure 1 illustrates a standard curve prepared with the use of this antiserum in the assay of 1:20,000, <sup>125</sup>I-labeled bovine PTH, and various concentrations of unlabeled bovine PTH. The B/F values are expressed as a percentage of initial B/F. Figure 1 also shows the percentage B/F values when (a) increasing volumes (1-20  $\mu$ l) of serum from a rat, obtained 48 hr after bilateral nephrectomy, and (b) increasing volumes (1-20  $\mu$ l) of pooled tissue culture medium, in which rat parathyroid glands were cultured for 48 hr, were added. It is apparent that the displacement curves for PTH standard, rat serum, and tissue culture medium from rat parathyroid glands are superimposable. In addition, by utilizing antiserum, appropriate changes are observed (data not shown) in serum PTH by hypocalcemia or hypercalcemia in the rat. Parathyroidectomized rats demonstrate undetectable serum levels of PTH. Basal serum PTH in the normal rats with this assay are 6.35 pg-equiv of bovine PTH/ml ( $n = 6$ ).

Serum calcitonin was determined by a method similar to the one developed in our laboratory for human and monkey CT. The assay utilizes an antibody developed against human synthetic CT in a goat. Human CT is also used as the tracer standard. Figure 2 illustrates a standard curve prepared with the use of this antiserum dilution of 1:20,000, <sup>125</sup>I-labeled human CT, and various concentrations of unlabeled human CT; the B/F values are expressed as a percentage of initial or trace B/F. Figure 2 also shows the percentage B/F values



1. Comparison of tracer displacement curves for purified bovine PTH, serum from a nephrectomized rat, and tissue culture medium from rat parathyroids. B/F values along the ordinate are expressed as percentage of the initial or trace B/F. Concentration along the abscissa are adjusted as shown to allow superimposition of one point of each curve to allow determination of similarity of curves. Each point represents mean  $\pm$  SD of six replicates in a single assay.

creasing volumes (1–70  $\mu$ l) of plasma from a calcium-infused rat and (b) increasing volumes (10–200  $\mu$ l) of an acetone–acetic acid extract of thyroid gland from a rat were assayed. It is apparent that the displacement curves for CT in human CT standard, rat plasma, and rat thyroid extract are superimposable. Basal serum CT levels in the normal rat this assay are 134 to 231 pg-equiv of human CT/ml ( $n = 22$ ) and there is a 2- to 3-fold increase in this value with calcium infusion ( $n = 8$ ). The levels of CT become detectable following thyroidectomy. Intra-assay coefficient of variation with this assay for normal pooled rat serum is 3.5%. All samples for parathyroid hormone and for parathyroid-related protein were analyzed in single assays. Serum calcium was determined by the EDTA titration method (13). Serum total protein was determined by refractometry (Bausch and Lomb Optical Corp., Buffalo, N.Y.). Group mean values for the experimental groups for a given time period were compared with those of the control group by Student's

**Results.** The animals tolerated the injection procedures and propranolol administrations well and gained weight normally. Initial weights were  $255 \pm 4$ ,  $254 \pm 5$ , and  $254 \pm 3$  g and the final weights at the end of the study were  $334 \pm 2$ ,  $334 \pm 3$ , and  $327 \pm 6$  g for groups I, II, and III respectively.

Figures 3 and 4 depict the changes in serum PTH and CT, respectively, in the rats receiving epinephrine, propranolol, or vehicle. There were no significant changes observed with time in either the serum PTH or CT levels in the vehicle-injected control rats. The concentrations of both serum PTH and CT were significantly increased in epinephrine-injected rats as compared to control animals at the end of 2 and 3 weeks, respectively, with further progressive increases during the remainder of the study. The maximum concentrations of PTH and CT were  $158 \pm 8$  and  $173 \pm 25\%$  of control, respectively, and were reached at the end of 5 weeks.

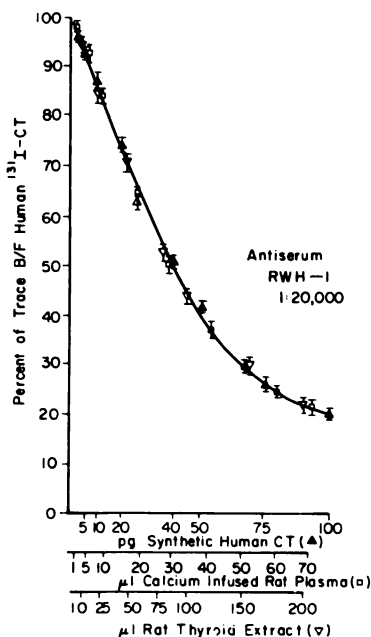


FIG. 2. Comparison of tracer displacement curves of synthetic human CT standard, serum from a calcium infused rat, and acetone acetic acid extract of a rat thyroid gland. B/F values along the ordinate are expressed as a percentage of the initial or trace B/F. Concentration scales along the abscissa are adjusted as shown to allow superimposition of one point of each curve to allow determination of similarity of curves. Each point represents the mean  $\pm$  SD of six replicates in a single assay.

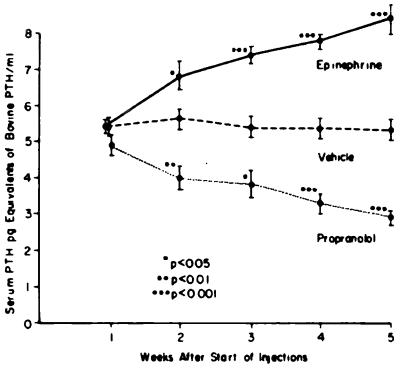


FIG. 3. Effect of administration of epinephrine, propranolol, or vehicle on serum parathyroid hormone concentration. Each point represents the mean  $\pm$  SE. The data are expressed in absolute values. See text for percentage changes.

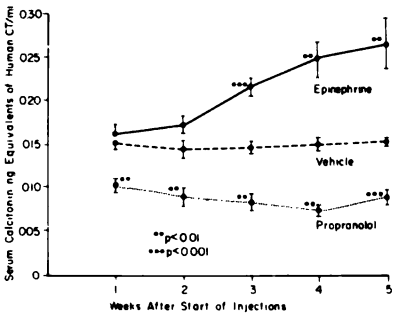


FIG. 4. Effect of administration of epinephrine, propranolol, or vehicle on serum calcitonin concentration. Each point represents the mean  $\pm$  SE. The data are expressed in absolute values. See text for percentage changes.

The concentrations of both serum CT and PTH were significantly decreased in rats receiving propranolol as compared to control animals at the end of 1 and 2 weeks, respectively, with further progressive decreases during the remainder of the study. The lowest concentrations for serum CT and PTH were  $49 \pm 4$  and  $54 \pm 5\%$  of control and were reached at the end of 4 and 5 weeks, respectively.

Figure 5 demonstrates that serum calcium values were not significantly different among the three groups at any time tested during the study.

Serum total proteins did not significantly change during the study in any of the groups.

**Discussion.**  $\beta$ -Adrenergic stimuli have been

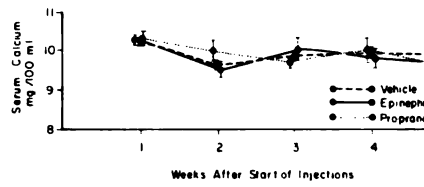


FIG. 5. Effect of administration of epinephrine, propranolol, or vehicle on serum calcium concentration. Each point represents the mean  $\pm$  SE.

shown to play a role in the secretion of PTH (1-5) and CT (6-8) in short-term studies. The present studies clearly demonstrate that long-term modification of  $\beta$ -adrenergic stimulation by administration of large doses of epinephrine and propranolol can also affect serum concentrations of these two hormones. In previous short-term studies, the stimulatory effects of isoproterenol and epinephrine on PTH and CT have been shown to be  $\beta$ -adrenergic as these could be blocked by propranolol (1, 4, 6).

The changes in the serum PTH and CT observed in the present studies were not due to hemoconcentration or hemodilution, as there was no change observed in the protein concentration. The present study does not entirely exclude the possibility that the changes observed in serum PTH and CT were not due to changes in their peripheral metabolism. However, epinephrine and propranolol can respectively stimulate or inhibit PTH secretion in *in vitro* studies (1). Therefore, it is likely that the changes observed in the concentrations of PTH in the present studies were because of changes in its secretion. The changes observed in the serum concentration of CT were also presumably because of changes in its secretion.

The lack of change in serum calcium concentration observed in the present studies may possibly be explained on the basis of simultaneous and comparable changes in both the PTH and CT, which have opposite effects on serum calcium concentration.

Previous case reports (14, 15) of patients with pheochromocytoma and evidence of excessive PTH production, one of whom had hypercalcemia (14), suggested that long-term excess of catecholamines may cause hyperparathyroidism. However, in subsequent studies, serum PTH levels were found to be normal in 10 unselected patients with

chromocytoma (16). The present studies show that, at least in the rat, long-term excess of catecholamines can increase serum PTH concentrations.

**Summary.** Injection of epinephrine to 250- to 300-g rats (0.3 mg/day for 2 weeks, followed by 0.6 mg/day for another 3 weeks) progressively increased the serum PTH and CT, whereas administration of approximately 40 mg of propranolol daily, in drinking water, progressively decreased the serum levels of both these hormones in comparison to control animals. The studies indicate that, similar to the short-term effects observed in previous studies, long-term modification of  $\beta$ -adrenergic stimuli can affect PTH and CT secretions.

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## The Effects of Ethanol on Cerebral Regional Acetylcholine Concentration and Utilization<sup>1</sup> (40330)

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The precise cerebral mechanism(s) of the acute effects of alcohol (ethanol) on the brain are still uncertain, but an alteration in neurotransmitter balance has been proposed as one possibility (1). Unfortunately, the available data concerning the level and metabolism of most key putative neurotransmitters following the acute and chronic administration of alcohol are conflicting (1). This may be due to species differences, dose of alcohol given, acute or chronic ethanol administration, brain area(s) assayed, and other methodologic difficulties.

The effect of acute and chronic alcohol exposure on cerebral acetylcholine (ACh) is controversial and incompletely defined (1). The aims of the present study were (i) to determine the effects of increasing acute oral doses of alcohol on regional cerebral ACh levels, (ii) to correlate the brain ACh concentrations with blood alcohol levels, (iii) to measure regional cerebral ACh utilization rates in rats at blood ethanol levels seen during modest human inebriation when rat brain ACh levels are essentially unaltered, and (iv) to assess the effects of prolonged oral alcohol consumption on brain ACh levels in rats. The results of these studies are the basis of this report.

**Experimental procedures.** Nonfasted female Sprague-Dawley rats, weighing 200 to 250 g, and female Swiss albino mice, weighing 20 to 25 g, were used for the acute alcohol and acetaldehyde experiments. Alcohol was diluted with saline to give a 25% solution (v/v) and was given by gavage to rats as a single oral dose of 3 to 7 g/kg body wt. Mice

received orally 20% ethanol as single 1.5 or 3 g/kg. Controls for the 3 g/kg dose received orally an equal volume of isocaloric glucose and were sacrificed at the appropriate time. Since glucose controls in these studies gave the same ACh values, in other acute experiments wherein net brain ACh levels were measured (Tables I and II) only saline controls were used. Acetaldehyde was dissolved in saline and was given to rats as 40 mg/kg intraperitoneally 15 min before sacrifice; this dose has previously been reported to lower brain ACh in mice (2).

For the chronic alcohol studies, Sprague-Dawley female rats weighing between 200 and 250 g were paired, one receiving ethanol and the second serving as a fed control. All rats were maintained on a 12-hr light-dark cycle in stainless-steel cages. They received the Lieber-DeCarli liquid diet containing either 6% (v/v) ethanol or isocalorically balanced maltose-dextrins fed controls as previously described. Rats were sacrificed after 5 weeks on alcohol. Growth curves and blood alcohol levels in these animals have been reported previously (3).

In both acute and chronic studies, ACh levels in the various brain regions were determined by pyrolysis-gas chromatography following head-focused microwave irradiation (5). The landmarks for identifying corpus striatum, midbrain, and brainstem have also been described by us earlier (4).

In order to estimate relative ACh turnover, the rate of decline of ACh levels following inhibition of ACh synthesis by hexamethonium-3 (HC-3) was determined. This decline of ACh has been shown to be dependent upon neuronal firing rate of cholinergic neurons (7-9) and therefore ap

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TABLE I. THE EFFECT OF ORAL ACUTE ALCOHOL ADMINISTRATION ON REGIONAL CEREBRAL ACETYLCHOLINE LEVELS IN RATS.<sup>a</sup>

Alcohol dose and time of sacrifice		Brain areas assayed				Blood alcohol level (mg/100 ml, mean $\pm$ SE)
		Cortex	Corpus striatum	Midbrain	Brainstem	
			(nmoles/g wet wt, mean $\pm$ SE)			
3 g/kg, 15 min	Alcohol (6)	27.4 $\pm$ 0.3	81.8 $\pm$ 2.0*	43.1 $\pm$ 1.4	29.6 $\pm$ 1.2	172 $\pm$ 14
	Saline control (5)§	26.1 $\pm$ 1.3	71.7 $\pm$ 4.3	40.2 $\pm$ 2.0	28.6 $\pm$ 2.1	—
	Glucose control (5)	26.1 $\pm$ 0.8	75.3 $\pm$ 2.7	41.9 $\pm$ 1.0	30.6 $\pm$ 0.4	—
3 g/kg, 30 min	Alcohol (5)	27.1 $\pm$ 1.0**	80.9 $\pm$ 4.1	37.5 $\pm$ 1.5	29.1 $\pm$ 0.4**	179.2 $\pm$ 16.0
	Saline control (5)§	23.7 $\pm$ 0.4	72.2 $\pm$ 1.9	38.3 $\pm$ 1.7	26.4 $\pm$ 0.7	—
	Glucose control (5)	24.9 $\pm$ 0.8	73.2 $\pm$ 4.8	38.0 $\pm$ 0.6	26.8 $\pm$ 0.8	—
3 g/kg, 150 min	Alcohol (5)	27.3 $\pm$ 1.2	79.4 $\pm$ 3.8	39.9 $\pm$ 0.6	28.2 $\pm$ 0.7	136 $\pm$ 13
	Saline control (6)§	26.0 $\pm$ 0.9	71.2 $\pm$ 2.6	39.7 $\pm$ 1.1	30.2 $\pm$ 1.4	—
	Glucose control (6)	24.6 $\pm$ 0.7	75.7 $\pm$ 3.2	41.0 $\pm$ 1.6	29.9 $\pm$ 1.8	—
1 g/kg, 15 min	Alcohol (5)	25.1 $\pm$ 1.0	90.7 $\pm$ 5.4**	37.3 $\pm$ 1.5	29.1 $\pm$ 1.9	219 $\pm$ 32
	Saline control (3)§§	22.2 $\pm$ 0.9	69.0 $\pm$ 1.3	35.8 $\pm$ 0.5	28.7 $\pm$ 0.9	—
1 g/kg, 15 min	Alcohol (4)	24.4 $\pm$ 1.2	87.3 $\pm$ 6.9**	38.3 $\pm$ 1.1	28.7 $\pm$ 0.8	278 $\pm$ 34
	Saline control (3)§§	22.2 $\pm$ 0.9	69.0 $\pm$ 1.3	35.8 $\pm$ 0.5	28.7 $\pm$ 0.9	—
1 g/kg, 15 min	Alcohol (5)	30.5 $\pm$ 1.0†	87.1 $\pm$ 2.8**	42.3 $\pm$ 2.5	31.4 $\pm$ 1.2	439 $\pm$ 31
	Saline control (3)§§	26.5 $\pm$ 1.3	70.1 $\pm$ 3.3	39.1 $\pm$ 2.3	29.4 $\pm$ 0.5	—
1 g/kg, 15 min	Alcohol (5)	33.5 $\pm$ 1.7**	101.5 $\pm$ 6.0**	43.9 $\pm$ 1.4†	31.2 $\pm$ 1.2	463 $\pm$ 30
	Saline control (3)§§	26.5 $\pm$ 1.3	70.1 $\pm$ 3.3	39.1 $\pm$ 2.3	29.4 $\pm$ 0.5	—

\* Statistical information:

†  $p < 0.05$ , one-tailed test.‡  $p < 0.05$ , two-tailed test.§  $p > 0.05$  vs three saline controls assayed on the same day but  $< 0.05$  vs pooled saline controls.||  $p < 0.05$ , one-tailed test, vs saline group ( $n = 3$ ) assayed on same day and  $< 0.05$ , two-tailed, vs all 45-min saline midbrain control data.§§ In rats receiving 3 g/kg alcohol, the saline and glucose values for each time interval and each area of brain were comparable ( $p > 0.05$ ) and were pooled ( $n = 10-12$ ) for statistical analysis.||| Saline groups for 4 and 5 g/kg alcohol and for 6 and 7 g/kg alcohol groups, respectively, were the same. In all instances where the saline controls for a given result consisted of only three rats assayed on the same day, comparison of the same alcohol data vs all pooled appropriate saline data ( $n = 17$ ) confirmed the statistical interpretation derived from the three saline controls alone.

TABLE II. THE EFFECT OF ACUTE ORAL ALCOHOL ADMINISTRATION ON CEREBRAL REGIONAL ACETYLCHOLINE LEVELS IN MICE.

Dose of alcohol	Brain region				Blood alcohol (mg/100 ml)
	Cortex‡	Corpus stri- atum (nmoles/g wet wt)	Midbrain	Brainstem	
1.5 g/kg (7)†	25.5 ± 1.4	56.9 ± 2.7*	28.5 ± 1.0	28.2 ± 1.2	134 ± 10
3 g/kg (7)†	35.1 ± 1.6*	68.3 ± 4.4*	38.2 ± 3.4*	28.1 ± 3.1	332 ± 17
Saline control (6)†	21.9 ± 0.9	47.4 ± 0.9	23.9 ± 2.2	27.7 ± 3.9	—

† Sacrifice 45 min after alcohol or saline administration.

‡ Mean  $\pm$  SE.\*  $p < 0.05$  vs saline controls.

be a valid index of cholinergic function. Briefly, rats were implanted with intraventricular polyethylene cannula as described by Robison *et al.* (10). Following 3 to 5 days of recovery, 20  $\mu$ g of HC-3 dissolved in water was administered to rats given ethanol (3 g/kg orally) or to controls which received isocaloric glucose. Previous studies (11) have

shown that this dose of HC-3 produces a linear decline in brain ACh in the areas studied over 45 min without mortality. The time of administration of HC-3 was varied relative to the time of alcohol administration so as to allow analysis of brain ACh at 0, 15, 30, and 45 min after HC-3 in both ethanol-treated rats and controls. The blood ethanol during

this period averaged  $170 \pm 17$  mg/100 ml (mean  $\pm$  SE). Declines in ACh in each brain region were converted into slopes by regression analysis, giving the relative turnover rate. Blood alcohol in rats and mice in the acute studies and in the chronic studies were measured by the alcohol dehydrogenase method (12).

Statistical analysis of brain ACh data in the acute studies was carried out by Student's *t* test, correlations between alcohol dose and ACh levels over the whole alcohol dose range by regression analysis, and the comparison of ACh slopes by analysis of covariance (13). The data were considered statistically significant with a *p* value of  $<0.05$ .

**Results.** Table I shows the effects of acute oral alcohol administration in various doses on the ACh concentration of several brain regions in rats. With 3 g of alcohol/kg body wt, blood alcohol levels were achieved at 45, 90, and 150 min, which roughly correspond to the concentration of alcohol considered legally intoxicating in man. With this dose, especially at 90 min when the mean blood alcohol was 179 mg/100 ml, cerebral regional ACh levels tended to be slightly higher than in controls, but this was not uniform in all brain areas and was statistically significant in only a few of them (wherein the control values tended to be lower). With increasing doses of alcohol of 4 to 7 g/kg the blood alcohol level rose progressively as one would expect ( $r = 0.945$ ,  $p < 0.001$ ) and brain ACh also tended to increase gradually (Table I). Again, however, the rise was modest and was

not present in all brain areas studied even at very high blood alcohol concentrations. For cortex and corpus striatum the relationship between alcohol dose and increase in ACh was significant ( $r = 0.53$ ,  $p < 0.001$  and  $r = 0.729$ ,  $p < 0.001$ , respectively) while the correlation for midbrain and brainstem was not significant ( $r = 0.265$ ,  $p = 0.118$  and  $r = 0.260$ ,  $p = 0.142$ , respectively). As is shown in Table II, doses of 1.5 and 3 g/kg of alcohol which gave mean blood alcohol levels of 134 and 332 mg/100 ml in mice at 45 min also tended to increase brain ACh levels and this was especially evident with the higher dose. There was no change in brainstem ACh with alcohol administration. By contrast, a single dose of acetaldehyde had no effect on regional brain ACh levels in rats (Table III). In addition, chronic administration of ethanol orally in a liquid diet for 5 weeks did not alter brain ACh concentration in rats (Table IV). This type of alcohol intake has been shown in previous studies to involve an average daily consumption of 3.7 ml of absolute alcohol per rat and gives blood alcohol levels of 70 to 200 mg/100 ml. At the time of brain assay for ACh (about 11:00 AM), with the animals fasted since 8:00 AM, the blood alcohol levels were essentially undetectable.

As a more sensitive index of possible derangement of ACh metabolism, the utilization rate of ACh was studied regionally in the brain at blood alcohol levels which coincide with human legal intoxication (approx 170 mg/100 ml) and which give essentially no evidence of alteration of net brain ACh in

TABLE III. THE EFFECT OF ACETALDEHYDE ON REGIONAL CEREBRAL ACETYLCHOLINE LEVELS IN RATS.

	Cortex*	Corpus striatum	Midbrain	Brainstem
Acetaldehyde† (7)	$24.5 \pm 0.8$	$75.7 \pm 3.1$	$39.6 \pm 1.9$	$28.8 \pm 1.1$
Saline (3)	$28.2 \pm 0.8$	$73.4 \pm 6.1$	$37.9 \pm 1.8$	$29.5 \pm 0.7$

† 40 mg/kg given iv. Rats sacrificed 15 min later. The acetaldehyde-injected rats had brain acetylcholine levels comparable to control values ( $p > 0.05$ ) (see also control values in Table I).

\* Mean  $\pm$  SE, nmoles/g wet wt.

TABLE IV. THE EFFECT OF CHRONIC† ALCOHOL INGESTION ON REGIONAL CEREBRAL ACETYLCHOLINE LEVELS IN RATS.

	Cortex*	Corpus striatum	Midbrain	Brainstem
Alcohol‡ (8)	$28.8 \pm 2.0$	$71.3 \pm 5.1$	$37.4 \pm 1.2$	$29.1 \pm 1.1$
Pair-fed control (8)	$28.9 \pm 0.8$	$69.2 \pm 3.5$	$39.4 \pm 0.9$	$29.9 \pm 1.7$

† Five weeks of oral alcohol intake (see Experimental Procedures).

‡ None of the alcohol values were statistically significantly different from appropriate control data.

\* Mean  $\pm$  SE, nmoles/g wet wt.

kg of ethanol at 45 min, Table I). In Table V, the rate of utilization was significantly decreased with this ethanol in the cortex and midbrain. Corpus striatum and brainstem tended to be lower in the alcohol group but did not reach statistical significance. *Conclusion.* The present study clearly shows in rats and mice that acute oral administration of ethanol increases significantly the utilization of ACh in some areas of the brain (Tables I and II). The changes, however, are only modest and occurred primarily at blood ethanol concentrations. Our data are in agreement with prior observations in rats with a single large dose of ethanol (14) but do not confirm the findings of Rawat (2) who found depressed ACh in whole brain of mice given 3 g/kg ethanol. This discrepancy may perhaps be explained by the use of brain tissue rapidly frozen in liquid nitrogen in our study (2); such a sacrifice procedure is known to result in partial degradation and indeed the levels of ACh in that tissue are less than half of those obtained by freeze fixation of brain. In our studies corpus striatum shows the most consistent decrease with ethanol, perhaps because of the most rapid ACh turnover rate (Tate and Ochoa, 1967). In the cortex and midbrain ACh, however, were also affected by alcohol, especially at higher blood alcohol levels. It is therefore, that the ethanol effect on ACh is a general one. The mechanism(s) by which ethanol may decrease brain ACh is still uncertain. Rawat (2) suggested that acetaldehyde generated from ethanol metabolism may combine with

sulphydryl groups of coenzyme A and thus decrease the precursor pool for ACh synthesis (2). In our studies, utilizing the same acetaldehyde protocol, no change in brain ACh was noted (Table III). Thus, while blood and brain acetaldehyde concentrations were not measured and it is possible that higher doses of acetaldehyde or administration of this drug over a prolonged time would exert some effect, our data with the single bolus of acetaldehyde do not support such a hypothesis. Against the acetaldehyde concept (2) are not only the observations that brain ACh increased, and not decreased, with alcohol administration but also the extensive *in vitro* and *in vivo* data with brain exposed to ethanol (15-17). In these studies, wherein no significant acetaldehyde is generated, alcohol inhibited the release of ACh from cerebral cortical slices and the mesencephalic reticular formation. These data clearly indicate that alcohol per se exerts an inhibitory effect on ACh release from brain. Our *in vivo* measurements of ACh utilization (Table V) (to our knowledge not previously carried out) showed a statistically significant decreased ACh turnover after ethanol administration in cortex and midbrain. In the corpus striatum and brainstem there was a tendency to a lower ACh utilization but this did not show statistical significance. This is consistent with the slight net accumulation of ACh in most of these areas with this low dose of alcohol. Conceivably at higher blood and brain alcohol levels a greater effect on ACh utilization would be shown. The changes observed here by us and by others (1) on brain ACh with ethanol are most consistent with the concept of Nikander and Wallgren (18) that alcohol

TABLE V. THE EFFECT OF ACUTE ORAL ALCOHOL ADMINISTRATION ON REGIONAL CEREBRAL ACETYLCHOLINE UTILIZATION.

	Control†	Alcohol†	Decrease in alcohol group (%)	p value
	(nmoles/g brain/min)			
Corpus striatum	0.43 ± 0.03 (33)*	0.30 ± 0.03 (32)	30.2	<0.001
Cortex	1.23 ± 0.08 (36)	1.09 ± 0.09 (38)	10.9	>0.10
Midbrain	0.54 ± 0.05 (35)	0.39 ± 0.03 (37)	28.3	<0.02
Brainstem	0.17 ± 0.05 (28)	0.10 ± 0.04 (31)	39.8	>0.10

† Given to rats as 3 g/kg orally while controls received isocaloric glucose in an equal volume of saline. All values are the mean ± SE turnover rate for the number of animals shown. For technique used to measure ACh see Experimental Procedures.

\* Number of samples assayed over 45 min, with 4 to 6 specimens at each time interval.

inhibits the action potential in brain. This may be mediated by a direct effect of alcohol on ionic conductance in the neuronal membrane (18, 19) and/or may be exerted at the presynaptic level (19). The net effect would be, as reported here, decreased utilization resulting in an accumulation of ACh. A precise quantitative stoichiometry between net ACh levels and its turnover, however, may not occur due to compartmentation of ACh in brain. No significant effects of alcohol on cerebral acetyltransferase or acetylcholinesterase activity have been reported (20). The effect of alcohol on brain ACh is not unique for this sedative and is shared by higher alcohols and barbiturates (19). Our observation (Table IV) that chronic alcohol intake does not alter brain ACh levels when alcohol is not present in blood implies that it is the presence of high concentrations of ethanol and not the duration of its administration which is relevant. An alternate interpretation, for which there are good data (17, 21), and which is not addressed by these studies, is that with chronic alcohol use the brain becomes insensitive or less sensitive to the effects of ethanol on ACh turnover.

The functional significance of ethanol-induced changes in brain ACh is uncertain. Erickson and Burnam (22) have shown that physostigmine shortens ethanol-induced sleep-time in mice and these studies have been confirmed by others (23). However, ethanol-induced EEG synchrony, an index of cerebral depression, could not be correlated with brain ACh changes after ethanol (16) and the use of various drugs which alter cerebral ACh status did not predictably alter behavioral depression (24). Finally, physostigmine appears to be a relatively nonspecific analeptic since it may reverse sedation induced by diazepam (unpublished observations) and other sedatives. Thus, the present study documents an increase of brain ACh and its decreased utilization with high levels of alcohol, but the functional significance of these findings remains to be established.

**Summary.** This study assessed the effect of alcohol, given as single increasing doses or chronically, on regional cerebral acetylcholine concentration. In the acute studies in both rats and mice, brain acetylcholine rose significantly, but modestly, at higher blood

ethanol concentrations. This effect was most consistent in the corpus striatum. At low blood alcohol levels, when brain acetylcholine levels were unaltered, the utilization rate of acetylcholine decreased in all brain areas and this was statistically significant in the cortex and midbrain. By contrast, in rats exposed to chronic oral ethanol intake but studied when blood alcohol was normal, brain acetylcholine was unaltered. These data are most consistent with the concept that alcohol directly depresses neuronal function resulting in decreased release (utilization) of acetylcholine and at high alcohol concentrations induces a modest accumulation of acetylcholine in brain.

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## Effect of Diet on Adhesion and Invasion of Microflora in the Intestinal Mucosa of Chicks<sup>1, 2</sup> (40331)

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Published results based on experiments with young chicks (1, 2) do not show conclusive evidence about active microbial participation in modifying the nutritional response of young chicks to a given diet. Our earlier work done in this laboratory (unpublished) with different cereal grains did, however, suggest that counts of microbes in the lumen of the gut differ with age and diet and, depending upon the diet, could be involved in the response of chicks to antibiotic-supplemented diets.

Adhesion of microflora to the intestinal wall in young pigs (3), man (4), and chicks (5) has been observed. The *Lactobacilli* are known to adhere to the epithelium of the crop and bursa in chicks soon after hatching, but no penetration into deeper tissues has been observed (6). Implantation of *Lactobacilli* through the oral route suppressed *Enterococci* in the small intestine and ceca and promoted growth in young chicks (7).

The present investigation was designed to characterize the nature and distribution of intestinal microbes which might adhere to the intestinal epithelium, penetrate the mucosa or become translocated to other organs. The growth response of young chicks on different diets, with and without supplemental penicillin, was also determined.

**Materials and methods.** Three replicate groups of 10 (five each of male and female) 1-day-old broiler chicks were randomly assigned to each of the eight different diets (Table I) under study. The chicks were

housed in electrically heated battery brooders with wire floors and free access to feed and water. Birds were reared up to 2 weeks of age, and effects of different diets on weekly body weights, feed consumption, and mortality were recorded and analyzed (8).

**Microbiological examinations of intestine and tissues.** At 10 days of age, three chicks from each treatment (one chick selected randomly from each of the three replicates) were fasted for 16 hr, sacrificed by electrocution, and immersed in a disinfectant (1% sepiisol) to minimize contamination of internal organs. The livers were then aseptically exposed, the surface of the right lobe was cauterized by a hot metal spatula, and samples were taken from the site (1.25 cm below the surface) with an inoculating loop for subsequent culturing on blood agar plates and incubation at 37° under aerobic and anaerobic conditions. Bacterial cultures were made similarly from the left kidneys. Bacterial isolates from livers and kidneys were identified by morphological characteristics and biochemical tests (9).

A section of small intestine (2.5 cm long) immediately below the yolk-stalk was removed without contaminating the exterior and transferred to preweighed sterile bottles containing 50 ml of phosphate-buffered saline, pH 7.1 (PBS). Each gut sample was opened with sterile scissors, washed with four changes of PBS (50 ml for the first washing, 10 ml for the subsequent washings), weighed, and then ground using a Thomas glass tissue grinder to make a 5% homogenate in sterile reinforced clostridial medium (RCM, BBL 11565) without agar. Serial dilutions of the fourth washing and the intestinal homogenates were made in liquid RCM, and each of at least five serial dilutions was plated in triplicate for bacterial counts. Pour plates of standard method agar (BBL 11638) containing 0.1% starch, 0.5% dextrose, and 5% horse blood and brilliant green bile agar (BBL

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ere used for enumeration of aerobes  
orms, respectively. RCM agar with  
horse blood (with second layer with-  
l) was used for culturing anaerobes  
bic jars with a Gaspak and catalyst  
Microbial adhesion to the intestinal  
s considered to have occurred if the  
of colony-forming units (CFU) re-  
from the homogenized intestine sig-

nificantly ( $P < 0.05$ ) exceeded that of the  
fourth washing (12).

*Results. Effects of diets on growth, feed,  
efficiency, and mortality.* The chicks fed a diet  
containing corn gained significantly higher  
body weights ( $P < 0.05$ ) than chicks fed diets  
containing either rye or beans (raw or  
cooked) (Table II). The chicks fed a diet  
containing rye grew significantly better ( $P <$

TABLE I. COMPOSITION OF DIETS AND OUTLINE OF THE EXPERIMENT.

Ingredients (%)	Treatment No.							
	1	2	3	4	5	6	7	8
Meal	22.09	22.09	22.09	22.09	3.7	3.7	3.7	3.7
Oil	5.00	5.00	5.00	5.00	5.3	5.3	5.3	5.3
1 bone meal	2.50	2.50	2.50	2.50	—	—	—	—
Key product	2.00	2.00	2.00	2.00	—	—	—	—
Red alfalfa	2.50	2.50	2.50	2.50	—	—	—	—
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Ice	0.73	0.73	0.73	0.73	1.00	1.00	1.00	1.00
n phosphate	1.00	1.00	1.00	1.00	2.00	2.00	2.00	2.00
premix <sup>a</sup>	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
premix <sup>b</sup>	0.05	0.05	0.05	0.05	0.10	0.10	0.10	0.10
ionine	0.04	0.04	0.04	0.04	0.35	0.35	0.35	0.35
at	—	—	—	—	7.00	7.00	7.00	7.00
tal diets								
	36.46	36.46	36.46	36.46	20.00	20.00	20.00	20.00
corn	63.54	63.54	—	—	—	—	—	—
	—	—	63.54	63.54	—	—	—	—
1 Mexican beans	—	—	—	—	80.00	80.00	—	—
ed Red Mexican	—	—	—	—	—	—	80.00	80.00
ed	—	—	—	—	—	—	—	—
penicillin (ppm)	—	0.50	—	0.50	—	0.50	—	0.50

n premix at 0.25% of the diet supplies the following per kilogram of the diet: vitamin A, 5500 I.U.;  
1650 I.C.U.; vitamin E, 4.4 I.U.; riboflavin, 3.3 mg; calcium pantothenate, 4.4 mg (or pantothenic acid,  
iacin, 22 mg; choline chloride, 577 mg; vitamin B<sub>12</sub>, 0.011 mg; and ethoxyquin, 62.2 mg.

il premix at 0.05% of the diet supplies the following per kilogram of the diet: Mn, 50 mg; Fe, 50 mg; Cu,  
0 mg; I, 1.5 mg; Ca, 60 mg; and Co, 0.5 mg.

exican beans were autoclaved at 1.06 kg/cm<sup>2</sup> of pressure for 30 min and oven-dried at 70°F.

exican beans (*Phaseolus vulgaris* Linneaus var. Othello) were used during these studies.

TABLE II. BODY WEIGHTS, FEED EFFICIENCY, AND MORTALITY OF CHICKS FED DIFFERENT DIETS.<sup>a</sup>

Diets	Average body weights (g)		Average feed effi- ciency (g)	Mortality (%)		
			Age			
	1 week	2 weeks		1 week	2 weeks	Total
Procaine penicillin	106 <sup>a</sup>	224 <sup>a, b</sup>	1.52 <sup>d</sup>	0	0	0
	108 <sup>a</sup>	232 <sup>a</sup>	1.47 <sup>d</sup>	0	0	0
	82 <sup>c, d</sup>	168 <sup>c</sup>	1.85 <sup>c, d</sup>	0	0	0
Procaine penicillin	91 <sup>b</sup>	199 <sup>b</sup>	1.49 <sup>d</sup>	0	0	0
	52 <sup>e</sup>	63 <sup>e</sup>	6.25 <sup>a</sup>	11	72	83
+ procaine penicillin	60 <sup>d</sup>	75 <sup>e</sup>	3.13 <sup>b</sup>	14	52	66
1 beans	78 <sup>d</sup>	131 <sup>d</sup>	2.30 <sup>c</sup>	2	0	2
1 beans + procaine penicillin	87 <sup>b, c</sup>	162 <sup>c</sup>	1.84 <sup>c, d</sup>	0	0	0

within each column followed by superscripts having common letters are not significantly different ( $P =$   
calculated with Duncan's multiple range test.



0.05) than the chicks fed a diet with raw beans. When autoclaved, the bean diet supported chick growth that was better ( $P > 0.05$ ) than that obtained with the diet containing rye, though it was still significantly lower ( $P < 0.05$ ) than that with the diet containing corn. Penicillin added at a 50 ppm level gave significantly higher body weights ( $P < 0.05$ ) over controls with diets containing rye or beans, but not in chicks fed a diet containing corn.

Birds on diets containing rye or autoclaved beans (Table II) were less efficient than those fed a corn diet. The efficiency of feed conversion was poorest in the chicks fed the diet containing raw beans during the same period. Replacing raw beans with autoclaved beans resulted in improved feed efficiency of chicks. Addition of penicillin to all of these diets markedly improved the feed efficiency.

During the 2-week period, 83% mortality was observed in chicks fed the diet containing raw beans (Table II). When procaine penicillin (50 ppm) was supplemented to this diet, mortality was reduced to 66%. In chicks fed the diet containing autoclaved beans, the mortality was only 2%, and supplement of penicillin to this diet prevented mortality completely. No mortality resulted in chicks fed diets containing corn or rye.

*Effects of diets on intestinal microbes.* In

chicks fed diets containing corn  $10^4$  aerobic organisms/g of wet sample from the lumen of the ileum were enumerated (Table III). Replacing rye with corn in the chick diet resulted in a significant decrease ( $P < 0.05$ ) in the viable counts of aerobes in lumen material of the gut and a significant increase ( $P < 0.05$ ) in the viable counts of aerobes adhered to the epithelial wall of the intestine. Feeding of diets containing raw beans to chicks significantly increased ( $P < 0.05$ ) the lumenal and epithelial counts of viable coliforms, total aerobes, and total anaerobes. Compared to raw beans feeding of autoclaved beans resulted in a significant decrease ( $P < 0.05$ ) in the viable counts of coliforms and total anaerobes in lumen and of those aerobes adhered to the epithelial wall. Supplementing the diets containing either corn or rye with penicillin (50 ppm) resulted in an insignificant ( $P > 0.05$ ) decrease in viable counts of total anaerobes (excluding coliforms) and total anaerobes. A significant decrease ( $P < 0.05$ ) in viable counts of coliforms and anaerobes was observed in chicks fed diets containing raw or autoclaved beans.

Adhesion of aerobes to the epithelial wall was not affected by feeding diets containing corn, while chicks fed diets containing rye or raw beans showed a significant increase ( $P < 0.05$ ) in adhesion of aerobes to the gut wall.

TABLE III. INFLUENCE OF DIFFERENT DIETS ON THE NUMBERS OF INTESTINAL BACTERIA FREE IN THE LUMEN AND ADHERED TO GUT WALL (ONE INCH BELOW YOLK-STALK) OF CHICKS AT TEN DAYS OF AGE.

Diets	No. of viable organisms/g of wet tissue (log)					
	Coliforms		Total aerobes		Total anaerobes	
	In lumen <sup>a</sup>	Adhered <sup>b</sup>	In lumen	Adhered	In lumen	Adhered
Corn	2.1 <sup>c</sup>	2.4	4.7	4.3	4.9	5.2
Corn + procaine penicillin	2.1	2.2	4.2	3.8	4.7	4.8
Rye	2.0	2.3	3.8	5.1	4.7	5.0
Rye + procaine penicillin	2.0	2.5	3.8	4.7	4.6	4.6
Raw beans	2.9	3.9	5.4	6.4	6.1	6.8
Raw beans + procaine penicillin	2.2	3.4	5.2	5.8	5.6	6.2
Autoclaved beans	2.1	2.9	5.1	5.1	5.4	6.0
Autoclaved beans + procaine penicillin	2.5	3.7	4.9	4.5	5.5	5.7

<sup>a</sup> In the fourth washing solution of the sampled intestine.

<sup>b</sup> In the 5% homogenates of the sampled intestine that had been washed four times.

<sup>c</sup> Least significant difference ( $P = 0.05$ ) for:

	Coliforms	Total aerobes	Total anaerobes
Means within each diet	0.5	0.62	0.44
Means within "in lumen" and "adhered" organisms in each diet	0.31	0.53	0.25

Means within each diet that differ by more than the stated value are significantly different ( $P = 0.05$  or less).

feeding of beans prior to feeding reduced adhesion of aerobes. No significant ( $P < 0.05$ ) adhesion of coliforms to the gut wall was evident in chicks fed diets containing corn or rye; however, feeding diets containing beans (raw or autoclaved) resulted in a significant increase ( $P < 0.05$ ) in adhesion of coliforms and total anaerobes. Supplementation of these diets with penicillin significantly ( $P < 0.05$ ) reduced the adhesion of aerobes (in chicks fed diets containing corn, rye, or autoclaved beans) and significantly ( $P < 0.05$ ) increased the adhesion of anaerobes in chicks fed the diet containing

beans isolated from the liver and kidney of chicks fed diets containing raw beans identified as *Escherichia coli* and *Streptococcus fecalis*. No bacteria were isolated from the livers and kidneys of chicks fed diets containing either corn or rye. No bacteria were observed in the liver and kidney of chicks fed the autoclaved beans.

**Discussion.** The results of the present study confirm earlier observations (1, 2) which indicated that the organisms adhering to or penetrating the gut wall might be significantly important to growth and survival. In the present study, the aerobes either adhered to or penetrated the ileal wall in chicks fed diets containing corn, rye or raw beans, and a major group of these adhering organisms was coliforms. Attachment of *E. coli* to the intestinal wall has been reported earlier (10-12). In chicks fed a diet containing raw beans, an increase in the number of anaerobes to the gut wall was

observed. Adhesion of aerobes (excluding coliforms) to the gut wall in the chicks fed the diet containing rye suggested that these microbes play a role in modifying the response of chicks to dietary antibiotics. MacAuliffe and Norris (13) obtained a much greater improvement with antibiotic supplementation in chicks fed a diet containing rye than to a similar diet containing corn.

The present results show that beans, when fed in the raw form in a chick diet, cause poor growth that is ameliorated by cooking the beans. These observations support earlier findings (14-17). Feeding autoclaved beans reduced adhesion of aerobes to the gut wall. The isolation of *E.*

*coli* and *S. fecalis* from the livers and kidneys of chicks fed diets containing raw beans is highly suggestive of microbial involvement in the heavy mortality observed in chicks on these diets and confirms our recent observation (17) that these organisms cause mucosal tissue damage, penetrate the epithelium and cause septicemia, organ invasion, and death. Feeding a diet containing autoclaved beans caused adhesion of coliforms to the intestinal wall, but no organisms were isolated from livers and kidneys. This indicates that the factors in raw beans which permit microbial penetration of the gut wall are heat-labile. Earlier Jayne-Williams and Hewitt (18) implicated strains of *E. coli* being responsible for the lethal effects of raw beans. Furthermore, they postulated that hemagglutinins (or possibly other heat-labile toxic factors) may interfere with normal body defense mechanisms, thereby allowing the normal intestinal bacteria to pass through lumen to other body tissues. The findings of the present study support the above hypothesis (19, 20). The increase in the microbial adhesion to the intestines of chicks fed diets containing either corn, rye or raw beans could be the result of lectin-mediated attachment of bacterial cells to the intestinal wall since lectins are known to combine with bacteria as well as intestinal mucosal cells. Our observations confirming it to be so will be reported in a separate publication.

A significant reduction in mortality of chicks (from 83% on diets containing raw beans to 2% on diets containing autoclaved beans) observed in this study is similar to that reported for Japanese quail (18). Penicillin added to the diet containing raw beans did not prevent mortality in chicks completely.

In our earlier work (unpublished) on the influence of dietary levels of raw beans on growth of chicks, a 46% dietary level resulted in 7% mortality versus 83% mortality in chicks fed at a level of 80% raw beans.

From the above observations and related earlier work in this laboratory (17), it is postulated that feeding diets containing raw beans causes the normal intestinal microflora to colonize on the intestinal wall in young chicks, and its magnitude is proportional to the level of raw beans in the diet. The more extensive colonization or damage to the in-

testinal mucosa due to the components of raw beans enables the microorganisms to become more invasive, as evidenced by the presence of aerobic organisms in the livers and kidneys. Further work on pathological examination of liver and kidney of chicks fed diets containing raw beans is in progress.

**Summary.** Compared to chicks fed a diet containing corn, those fed a diet containing rye showed significantly lower growth that was ameliorated by antibiotic supplement to the diet. Adhesion of aerobes (excluding coliforms) and anaerobes to the intestinal wall was indicated in the chicks fed the diet containing rye which was reduced by penicillin supplementation. There was no mortality in chicks fed diets containing corn or rye, whereas the poorest growth and a very high mortality resulted in chicks fed diets containing raw beans. Such adverse effects were alleviated by dietary antibiotic supplement. High numbers of aerobes, mainly coliforms, were found adhered to the mucosal wall of the chicks fed diets containing raw beans, and *E. coli* and *S. fecalis* organisms were isolated from their kidneys. Autoclaving the beans greatly improved growth, reduced mortality, and caused no adhesion of intestinal aerobes to the mucosal wall. A penicillin supplement to the diet resulted in further improvement of growth and reduction of mortality.

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## The Effect of Prostaglandin E<sub>2</sub> and Indomethacin on the Placental Vascular Response to Norepinephrine<sup>1</sup> (40332)

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Various studies provide indirect or direct evidence that prostaglandin E<sub>2</sub> is involved in regulating the maternal placental blood flow (1-5). Terragno *et al.* (1), using anesthetized pregnant dogs, and Venuto *et al.* (2), using anesthetized pregnant rabbits, have demonstrated: (a) that the uteroplacental unit is a rich source of prostaglandin E-like material and (b) that the blockade of prostaglandin synthesis is accompanied by a decrease in the uterine blood flow and a decrease in the concentration of prostaglandin E-like material in the uterine venous blood.

Direct evidence comes from a study previously reported by this laboratory using near-term pregnant sheep (3). In this study the injection of 20 µg/kg prostaglandin E<sub>2</sub> directly into the maternal circulation increased the placental vascular resistance. This increase in placental vascular resistance was due to the uterine contraction induced by prostaglandin E<sub>2</sub> which masked the effect of prostaglandin E<sub>2</sub> on the placental vasculature. When this effect of prostaglandin E<sub>2</sub> on the noncotyledonary uterus was bypassed by administering the drug via the fetal venous catheter, there was a small but significant increase in placental blood flow.

Thus, there appears to be evidence supporting the involvement of prostaglandins in the maintenance of placental blood flow. The mechanisms by which prostaglandins contribute to the control of placental blood flow are not clear. Many investigators have demonstrated that prostaglandins can regulate regional blood flow in a variety of vascular beds by modifying their reactivity to adrenergic stimuli (6-10). However, the modulation

of the vascular response to adrenergic stimuli by prostaglandins varies greatly both quantitatively and qualitatively depending upon the species or vascular bed studied. It was therefore the purpose of this study to investigate the possibility that prostaglandins may influence the regulation of blood flow in the near-term ovine placenta by altering the response of the vasculature to catecholamines.

**Methods.** Eleven pregnant sheep were surgically prepared between Day 125 and Day 135 of gestation. The jugular vein was catheterized and the sheep was sedated with sodium pentobarbital (Nembutal, 10 mg/kg) and a spinal anesthetic (Xylocaine). Xylocaine (3%) was injected subcutaneously in the ventral cervical region to serve as a local anesthetic during the placement of the left ventricular catheter via the carotid artery. The left ventricular catheter consisted of a polyethylene catheter (i.d. 1.6 mm, o.d. 2.0 mm) within which was threaded a polyvinyl catheter (i.d. 0.7 mm, o.d. 1.2 mm) which extended 1 cm from the tip. Correct placement of the left ventricular catheter was confirmed by monitoring the blood pressure recording. A polyvinyl catheter was inserted in a superficial artery of the maternal hindlimb and advanced 20 cm into the femoral artery. In order to monitor amniotic fluid pressure a catheter was secured to the fetal hindlimb via a midline incision in the maternal abdomen. The femoral and amniotic catheters were secured on the side of the abdomen. The left ventricular and jugular catheters were encased in a gauze bandage which was tucked under an elastic bandage wrapped around the neck. The ewes were injected with 200,000 units of penicillin following surgery.

The experiments were performed 2 days after surgery with the ewe standing quietly in a stanchion in the laboratory. At this time the maternal arterial pH of all sheep was not less

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than 7.4. All pressures were monitored with Statham P23Db transducers positioned at the level of the scapulo-humeral joint and recorded by an R411 Beckman recorder. The placental blood flow was measured using the radioactive microsphere technique in which the microspheres were injected into the left ventricle while simultaneously withdrawing an integrated arterial sample from the femoral catheter at the rate of 2.06 ml/min using a Harvard infusion pump as previously described (11). The microspheres (3M Co., New England Nuclear) had a mean diameter of 25  $\mu\text{m}$  and were labeled with one of the following isotopes:  $^{125}\text{I}$ ,  $^{109}\text{Cd}$ ,  $^{57}\text{Co}$ ,  $^{46}\text{Sc}$ , or  $^{85}\text{Sr}$ . Organ blood flows were measured with the use of microspheres rather than electromagnetic flow probes because the microsphere technique allows the separation of the uteroplacental blood flow, which is measured by the flow meter method, into the individual placental and nonplacental components.

The protocol for all experiments was to measure the blood flow before (control) and 1.5 min after (test) the left ventricular injection of 1  $\mu\text{g/kg}$  norepinephrine (Levophed, Winthrop). The response to norepinephrine was measured 1.5 min following norepinephrine injection because of observations made in pilot experiments in which a uterine arterial flow probe was employed. Uteroplacental blood flow was found to be relatively stable and depressed maximally at 1.5 min post-norepinephrine injection. The animal was allowed to return to control conditions and one of two additional procedures was then performed.

(1) *Pretreatment with prostaglandin  $E_2$* . In this series of experiments prostaglandin  $E_2$  was infused continuously into the jugular catheter at the rate of 20  $\mu\text{g/min}$ . Ten minutes after the start of prostaglandin  $E_2$  infusion, blood flows were measured before and after the injection of norepinephrine as previously described. Amniotic fluid pressure was monitored throughout the experiment. If the infusion of prostaglandin  $E_2$  caused an increase in amniotic fluid pressure, the infusion rate was decreased to 10  $\mu\text{g/min}$ . This was done in animal number 2. Five sheep were used in this series.

(2) *Pretreatment with indomethacin*. Indomethacin was used to inhibit endogenous

prostaglandin synthesis. Venuto *et al.* (2) have reported that the intravenous infusion of 2 mg/kg indomethacin significantly decreased uterine venous prostaglandin  $E_2$  concentration in pregnant rabbits. In this series of experiments, 10 mg/kg indomethacin (Sigma) dissolved in dimethyl sulfoxide (100 mg/ml) was infused into the jugular catheter at a rate of 0.5 ml/min. Twenty minutes later the blood flows were measured before and after the injection of norepinephrine as previously described. Six sheep were used in this series.

At the end of the experiments the ewes were sacrificed and the uterus and contents were removed. The fetus and fetal membranes were removed from the uterus. The placental cotyledons were dissected free from the remaining uterine tissue and were prepared and analyzed for radioactivity in a three-channel Nuclear Chicago 1185 gamma counter in a manner previously described (11). Counts per minute obtained from the gamma counter output were reduced to the number of spheres contained in the sample. Placental blood flows were calculated by the following equation from Makowski *et al.* (12):

$$\text{placental flow (ml/min)} = \frac{\text{spheres in organ/spheres in reference arterial sample}}{\text{(withdrawal rate)}}$$

The resistance was calculated by dividing the mean maternal arterial pressure by the blood flow.

The changes in vascular resistance in response to norepinephrine were expressed as resistance ratios. The resistance ratios were defined as the ratio of the resistance seen in the test condition 1.5 min after norepinephrine injection, to the resistance seen in the control condition. The paired *t* test was used to determine the significance of differences between means. Data are reported as means  $\pm$  standard errors of the mean.

*Results. Part 1: The effect of prostaglandin  $E_2$  infusion on the vascular response to norepinephrine.* The effect of norepinephrine on the arterial blood pressure, placental flow, and vascular resistance of five sheep before and after pretreatment with prostaglandin  $E_2$  is given in Table I. Twins occurred in sheep 3 and 4. In these cases the placentas serving each fetus were analyzed separately. The in-

n of norepinephrine increased the arterial blood pressure by 22% ( $P < 0.02$ ). When

animals were pretreated with prostaglandin  $E_2$  the injection of norepinephrine raised the blood pressure by 14% ( $P < 0.03$ ).

The injection of norepinephrine decreased the placental blood flow by 35% ( $P < 0.03$ ). After pretreatment with prostaglandin  $E_2$  the injection of norepinephrine decreased the placental flow by 17% ( $P < 0.03$ ). In Table I, norepinephrine injection raised the placental vascular resistance by 48% ( $P < 0.03$ ). With prostaglandin  $E_2$  present the injection of norepinephrine increased the placental resistance by 48% ( $P < 0.03$ ).

Expressing these changes in placental vascular resistance in response to norepinephrine in terms of resistance ratios, we found a resistance ratio of  $2.27 \pm 0.52$  after prostaglandin  $E_2$  pretreatment. After pretreatment with prostaglandin  $E_2$ , we obtained a resistance ratio of  $1.47 \pm 0.21$ . This

depression of the resistance ratio was significant ( $P < 0.03$ ).

In the present study we observed that the continuous infusion of 20  $\mu\text{g}/\text{min}$  prostaglandin  $E_2$  for 10 min caused (a) no change in the maternal blood pressure, (b) a decrease in the placental blood flow, and (c) an increase in the placental vascular resistance.

*Part 2: The effect of indomethacin on the placental response to norepinephrine.* The effect of pretreatment with indomethacin on the maternal responses to norepinephrine in six sheep is given in Table II. The injection of norepinephrine increased the arterial blood pressure by 9% ( $P < 0.007$ ). When the animals were pretreated with indomethacin the injection of norepinephrine increased the blood pressure by 20% ( $P < 0.005$ ). The injection of norepinephrine decreased the placental blood flow by 44% ( $P < 0.02$ ). Following the pretreatment with indomethacin the injection of norepinephrine decreased

TABLE I. THE EFFECT OF PRETREATMENT WITH 20  $\mu\text{g}/\text{min}$  PROSTAGLANDIN  $E_2$  ( $\text{PGE}_2$ ) ON MEAN ARTERIAL PRESSURES, PLACENTAL BLOOD FLOW, AND VASCULAR RESISTANCES BEFORE (C) AND 1.5 min AFTER (T) THE INJECTION OF 1  $\mu\text{g}/\text{kg}$  NOREPINEPHRINE IN FIVE NEAR-TERM SHEEP.

No.	Mean arterial pressures (mm Hg)				Placental blood flow (ml/min)				Placental resistance (mm Hg $\times$ min)/ml			
	Before $\text{PGE}_2$		After $\text{PGE}_2$		Before $\text{PGE}_2$		After $\text{PGE}_2$		Before $\text{PGE}_2$		After $\text{PGE}_2$	
	C1	T1	C2	T2	C1	T1	C2	T2	C1	T1	C2	T2
95	134		95	115	921	477	627	499	0.103	0.281	0.152	0.230
98	102		90	95	1226	1030	873	927	0.080	0.099	0.103	0.102
100	116		92	107	761	549	590	506	0.131	0.211	0.156	0.211
					645	446	511	402	0.155	0.260	0.180	0.266
90	110		99	101	411	290	355	312	0.204	0.379	0.279	0.324
					530	414	496	433	0.170	0.266	0.200	0.233
100	128		105	128	704	174	552	254	0.142	0.736	0.190	0.504
97	118		96	109	747	483	572	476	0.141	0.319	0.180	0.267
	$\pm 1.9$	$\pm 5.8$	$\pm 2.7$	$\pm 5.7$	$\pm 99.2$	$\pm 106.6$	$\pm 59.9$	$\pm 82.9$	$\pm 0.016$	$\pm 0.076$	$\pm 0.020$	$\pm 0.047$
	$P < 0.02$		$P < 0.02$		$P < 0.003$		$P < 0.03$		$P < 0.03$		$P < 0.04$	

Sheep 3 and 4 had twin fetuses, in which case the uterine horns and placentas serving each fetus were analyzed separately.

TABLE II. THE EFFECT OF PRETREATMENT WITH 10 mg/kg INDOMETHACIN (INDO) ON MEAN ARTERIAL PRESSURES, PLACENTAL BLOOD FLOWS, AND VASCULAR RESISTANCES BEFORE (C) AND AFTER (T) INJECTION OF 1  $\mu\text{g}/\text{kg}$  NOREPINEPHRINE IN SIX NEAR-TERM SHEEP.

No.	Mean arterial pressures (mm Hg)				Placental blood flow (ml/min)				Placental resistance (mm Hg $\times$ min)/ml			
	Before INDO		After INDO		Before INDO		After INDO		Before INDO		After INDO	
	C1	T1	C2	T2	C1	T1	C2	T2	C1	T1	C2	T2
95	100		100	125	954	583	1003	450	0.100	0.172	0.110	0.278
106	110		116	124	1642	996	1447	794	0.065	0.110	0.080	0.156
90	98		94	104	408	332	312	247	0.221	0.295	0.301	0.421
112	119		112	126	461	378	355	305	0.243	0.315	0.315	0.413
110	120		118	155	953	604	937	546	0.115	0.199	0.126	0.284
80	100		93	125	1179	259	874	173	0.068	0.389	0.106	0.723
99	108		106	127	933	525	821	419	0.135	0.247	0.173	0.379
	$\pm 5.2$	$\pm 4.1$	$\pm 4.6$	$\pm 6.7$	$\pm 188.2$	$\pm 109.7$	$\pm 174.8$	$\pm 93.2$	$\pm 0.032$	$\pm 0.042$	$\pm 0.043$	$\pm 0.080$
	$P < 0.007$		$P < 0.005$		$P < 0.02$		$P < 0.01$		$P < 0.03$		$P < 0.03$	

the placental flow by 49% ( $P < 0.01$ ). As seen in Table II, the injection of norepinephrine caused an increase in the vascular resistance of the placenta by 83% ( $P < 0.03$ ). With indomethacin pretreatment the injection of norepinephrine increased the placental resistance by 119% ( $P < 0.03$ ). When these changes in placental vascular resistance in response to norepinephrine were expressed in terms of resistance ratios, we found a resistance ratio of  $2.25 \pm 0.70$  without indomethacin pretreatment. After pretreatment with indomethacin, the resistance ratio was  $2.71 \pm 0.84$ . This increase in the resistance ratio was significant ( $P < 0.03$ ).

The infusion of indomethacin caused a significant increase in placental vascular resistance of 28% ( $P < 0.02$ ).

**Discussion.** Prostaglandins are lipids which are produced by most cells and appear to act locally (13). These characteristics have made their physiologic actions difficult to describe. There are two aspects to prostaglandin action: (i) the substance may act directly and (ii) they may act to modify the action of other agents. Several investigators have attempted to delineate the role of prostaglandins in the maintenance of vascular homeostasis by describing how exogenous prostaglandins modify the action of systemic vasoactive agents and how the blockade of endogenous prostaglandin synthesis modifies the action of systemic vasoactive agents (8, 14). These observations have been made either using nerve stimulation or exogenous norepinephrine as the primary stimulus. The action of the primary stimulus on the organ in question is observed in the control condition, after the infusion of prostaglandin  $E_2$  and after the infusion of indomethacin. Using this rationale Malik and McGiff (8) have shown that indomethacin potentiates the response of the rabbit kidney to norepinephrine and Fink *et al.* (14) have shown that prostaglandin  $E_2$  depresses the response of the rabbit kidney to norepinephrine. These results have led these investigators to postulate that prostaglandins play a role in the maintenance of vascular homeostasis in this organ. In the work described here we are concerned only with the rationale and logic behind this type of approach. There is considerable controversy over the factors which regulate renal blood flow and the actual re-

sults that are obtained vary depending species and preparation used. While factors may differ as to the role that endogenous prostaglandins play in the renal circulation they appear to be united in the approval of the logical sequence behind the design of the experiments such as those described above.

Previous studies have shown indirect evidence that endogenous prostaglandins may be involved in the regulation of the blood flow in the pregnant uterus (1, 2). In an attempt to obtain direct evidence of this action we have used the above rationale to the study of the placental vascular bed in near-term sheep. We have used exogenous norepinephrine as the primary stimulus and have attempted to modulate the response of the uterine vasculature to the stimulus with exogenous prostaglandin  $E_2$  and with indomethacin.

When the placental vascular bed was pretreated with prostaglandin  $E_2$  we observed an increase in vascular resistance which confirms a previous result from this laboratory (3) which was postulated at that time due to the ability of prostaglandin  $E_2$  to induce a uterine contraction. In the first series of experiments we observed that pretreatment with prostaglandin  $E_2$  significantly depressed the placental response to norepinephrine. In the second series of experiments we observed that pretreatment with indomethacin caused a significant increase in the uteroplacental vascular resistance. We also observed that indomethacin significantly increased the placental response to norepinephrine. These data support the conclusions that the placental vascular bed synthesizes prostaglandins and that these substances can suppress the response of that vascular bed to exogenous norepinephrine.

**Summary.** The vascular response to norepinephrine is expressed in terms of a resistance ratio which is defined as the ratio of placental vascular resistance seen 1.5 min after norepinephrine administration to that seen before norepinephrine administration. The infusion of  $1 \mu\text{g/kg}$  of norepinephrine to near-term sheep significantly increased the vascular resistance of the placenta to a ratio of  $0.52$  (mean  $\pm$  SEM;  $N = 7$ ). Pretreatment with  $20 \mu\text{g}$  of prostaglandin  $E_2$  per kilogram significantly decreased the placental re-

to norepinephrine to a resistance ratio of  $1.47 \pm 0.21$  which was 65% of the untreated response ( $N = 7$ ). Pretreatment with 10 mg/kg indomethacin significantly increased the placental response to norepinephrine from a resistance ratio of  $2.25 \pm 0.70$  to  $2.71 \pm 0.84$ , which is 120% of the untreated value ( $N = 6$ ). Prostaglandin  $E_2$  attenuated the placental vascular response to norepinephrine and indomethacin potentiated this response.

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# Relation of Vitamin D-Dependent Intestinal Calcium-Binding Protein to Calcium Absorption during the Ovulatory Cycle in Japanese Quail (40333)

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The concentration of the vitamin D-dependent calcium-binding protein (CaBP) in the small intestine correlates with the degree of vitamin D-mediated intestinal absorption of calcium with few exceptions (1). One presumed exception was from studies with the laying hen and laying Japanese quail. In both of these species, it was noted that the absorption of calcium was greater during the period when the eggshell was undergoing calcification than when no eggshell was being formed; however, the amount of CaBP in the intestinal mucosa did not change correspondingly (2-4). It was proposed by Bar, Hurwitz, and colleagues (2-4) that there exists in the laying bird a rapidly modulating calcium transport mechanism not associated with CaBP.

In evaluating the relation between CaBP concentrations and calcium absorption, critical consideration must be given to the method by which calcium absorption is measured. In the Bar-Hurwitz experiments, use was made of a nonabsorbable indicator method that measures *net* calcium absorption. Distinct from this are procedures that measure the unidirectional movement of calcium from intestine to blood using a radiotracer of calcium. The latter gives an estimate of the *efficiency* of the calcium absorptive mechanisms, which better correlates with CaBP concentrations than would net calcium absorption.

The present experiment was undertaken to determine if there is, in fact, a change in the efficiency of calcium absorption during the egg-laying cycle in Japanese quail. The results indicate no significant difference in calcium translocation across the intestine as a function of eggshell formation.

**Methods.** Japanese quail in the egg-laying stage were individually housed and periodicity of oviposition was recorded for each bird. Calcium absorption was measured in quail forming an eggshell (12-17 hr after

oviposition verified by intrauterine presence of an egg) and in quail not forming an eggshell (1-2 hr after oviposition). For the measurement of the absorption of calcium, quail were anesthetized with ether, a laparotomy was performed, and a 0.5-ml dose of  $^{47}\text{Ca}$  (1 mM  $\text{CaCl}_2$ , 150 mM  $\text{NaCl}$ , pH 7.4, 0.1  $\mu\text{Ci}$   $^{47}\text{Ca}$ ) was injected into the lumen of the ligated loop of duodenum. The loop was replaced into the peritoneal cavity and the incision was closed with wound clips. After 15 min, the quail were bled by heart puncture and then they were killed with an overdose of nembutal. The duodenal loop was excised and counted immediately for residual  $^{47}\text{Ca}$  activity using a gamma scintillation detector with a single-channel analyzer set to eliminate any contribution from the  $^{47}\text{Sc}$  daughter. After the gut loop was counted, the residual contents in the lumen were removed by rinsing, the loop was cut open and scraped, and the concentration of CaBP in the intestinal mucosa was determined by a radial immunoassay, as previously described (5). The tibiae were also excised and counted for  $^{47}\text{Ca}$ .

The calcium content of the plasma was determined by atomic absorption spectrometry, and plasma phosphorus by the Fiske-Subbarow method (6).

Calcium absorption is expressed as a percentage of administered dose, and CaBP as micrograms per milligram of total soluble protein. Protein was determined by the Lowry procedure (7).

**Results.** The data in Table I indicate that there were no significant differences ( $P > 0.05$ ) in any of the measured parameters between those Japanese quails in which eggshells were being calcified and in those quail in the noncalcifying stage. The only exception was body weight ( $P < 0.025$ ) which undoubtedly reflects the presence or absence of the forming egg in the body cavity.

**Discussion.** The present finding that the

I. RELATION OF INTESTINAL CaBP AND DUODENAL CALCIUM ABSORPTION TO THE EGG-LAYING CYCLE OF JAPANESE QUAIL.<sup>a,b</sup>

Group	Body weight (g)	Duodenal absorption of <sup>47</sup> Ca (% dose)	<sup>47</sup> Ca in tibia (% dose)	Intestinal CaBP (μg/mg of protein)	Plasma	
					Ca (mg/100 ml)	P <sub>i</sub> (mg/100 ml)
noncalcifying (9)	128 ± 3	52.1 ± 6.8	0.99 ± 0.08	32.8 ± 3.2	14.2 ± 1.4	5.2 ± 0.7
calcifying (9)	139 ± 3	46.1 ± 3.0	1.00 ± 0.04	32.5 ± 2.3	17.4 ± 0.9	6.8 ± 0.8

values represent the means ± standard errors of the mean of nine birds per group.

<sup>a</sup> Some of the values for any parameters were significantly different from one another at  $p > 0.05$  except body weight ( $p < 0.025$ ).

ion of calcium does not change as a function of eggshell formation is in apparent agreement with the information previously reported by Bar, Hurwitz, and colleagues. However, the disparity is more likely conceptual than real. The significant difference between the two studies is the manner in which calcium absorption was measured. As alluded to previously, the Hurwitz technique measures net calcium absorption and, by this procedure, the net absorption of calcium was observed to be higher in the quail during the period of eggshell formation than during the period when no eggshell was being formed. This is possible since the forming eggshell constitutes a significant calcium "sink" into which absorbed calcium is deposited and, thus, less calcium is available for return to the intestinal lumen. When no eggshell is in the formative stage, more of the absorbed calcium can be reabsorbed and is not secreted into the intestinal lumen, resulting in a decrease in net calcium absorption.

The procedure used in the present study, in which measurement of calcium absorption is independent of the subsequent fate of the absorbed calcium. Over the 15-min absorption period, the amount of absorbed <sup>47</sup>Ca that remains in the intestinal tract is negligible and, under these conditions, no difference in <sup>47</sup>Ca absorption was detected between the different stages of the egg-laying cycle.

The conclusion is offered that the efficiency of calcium absorption does not change during the calcifying stage and the noncalcifying stage of the egg in Japanese quail. This finding is consistent with the observation that kidney 25-hydroxycholecalciferol-1-hydroxylase activity does not differ during egg formation and in the noncalcifying period, up to 4 hr after ovulation (4, 8).

Thus, there appears to be a reasonable correlation between the intestinal transport of calcium, intestinal CaBP levels, and the activity of the kidney-1-hydroxylase enzyme system in this physiological state, and the proposal that the laying quail contains a rapidly modulating, non-CaBP-dependent, calcium-absorptive mechanism appears to be unwarranted.

**Summary.** Duodenal CaBP levels and the efficiency of <sup>47</sup>Ca absorption by the duodenal segment of ovulating Japanese quail were determined as a function of eggshell formation. No differences in these parameters were noted in quail in which eggs were being calcified and in quail with no egg in the calcification stage. Thus, there is a correlation between the efficiency of Ca absorption and the level of vitamin D-dependent intestinal calcium-binding protein in this physiological state in Japanese quail.

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## Synthesis of Rat Liver Mitochondrial Proteins after the Administration of a Nonlethal Dose of Cycloheximide (40334)

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Since the earliest report that mitochondria incorporate labeled amino acids into polypeptides *in vitro* (1), major efforts have been directed toward the isolation and characterization of mitochondrially synthesized polypeptides (2-4). From carefully designed *in vitro* systems, a few of the products of mitochondrial protein synthesis have been identified. Most of these proteins were low molecular weight, hydrophobic, chloroform-methanol-extractable inner-membrane proteins (5-12). Understanding of the mitochondrial protein synthetic system has also been aided by the use of cycloheximide and chloramphenicol in *in vitro* and *in vivo* systems (13-16). Most often studies with cycloheximide *in vivo* were carried out with lethal doses (10-100 mg/kg) which cause irreversible metabolic and cellular changes (17-20). Thus, it is difficult to distinguish normal physiological events from toxic effects of the antibiotic. With a nonlethal dose of cycloheximide (2 mg/kg) we have demonstrated that the incorporation of radioactive label into low molecular weight mitochondrial trichloroacetic acid-insoluble material is stimulated in the absence of cytoplasmic protein synthesis whereas the synthesis and/or incorporation of large cytoplasmically synthesized proteins into mitochondria requires the presence of cytoplasmic protein synthesis. The differential labeling patterns of these mitochondrial proteins presented in this report extend the cooperative nature of cytoribosomal and mitoribosomal proteosynthetic systems observed with mammalian cells in culture to the intact rat.

**Materials and Methods.** The experiments were performed on male Wistar rats (210  $\pm$  10 g). Maintenance and treatment of the animals were carried out as previously described (21). Mitochondria (3 $\times$  washed) of the control and cycloheximide-treated rat liver were isolated in separate tubes under identical conditions according to the procedure described (15).

Extractions of mitochondrial proteins performed with 0.05 M Na<sub>2</sub>HPO<sub>4</sub> buffer at various pH values containing 0.05 M  $\beta$ -mercaptoethanol. The specific pH values produced by the dropwise addition of sodium hydroxide (5 N) or concentrated phosphoric acid to 0.05 M  $\beta$ -mercaptoethanol in 0.05 M Na<sub>2</sub>HPO<sub>4</sub>. Proteins were extracted sequentially with buffers of decreasing pH values (7.5, 6.5, 5.5, 4.5) and with buffers of increasing pH values (8.5, 9.5, 10.5, 11.5). After each extraction, the pellet was stirred for 10 min in the appropriate buffer and centrifuged for 30 min at 27,000 g. The pellet from each series (4.5P and 11.5P) was solubilized in 1% SDS/0.1 M  $\beta$ -mercaptoethanol/10 mM Tris-HCl buffer, pH 7.5, to 100 $^\circ$  and dialyzed against 0.01 M sodium phosphate buffer (pH 7.2) containing 0.1%  $\beta$ -mercaptoethanol and 0.1% SDS. After dialysis, separation of proteins was carried out immediately on 10% polyacrylamide gels containing 0.1% SDS according to the procedure of Dehlinger and Schimke (22).

For amino acid incorporation, groups of four animals were injected ip with [<sup>3</sup>H]leucine (40-60 Ci/mmol) or <sup>3</sup>H-labeled protein hydrolysate (mixture 3130-08, Schwarz/Mann) 1 hr before sacrifice. Samples containing radioactivity were determined as described by Ch'ih *et al.* (23). Protein was determined by the method of Lowry *et al.* (24). To eliminate interfering substances such as  $\beta$ -mercaptoethanol, all samples were treated with 10% chloroacetic acid and the precipitates redissolved in 0.1 N NaOH before protein determination.

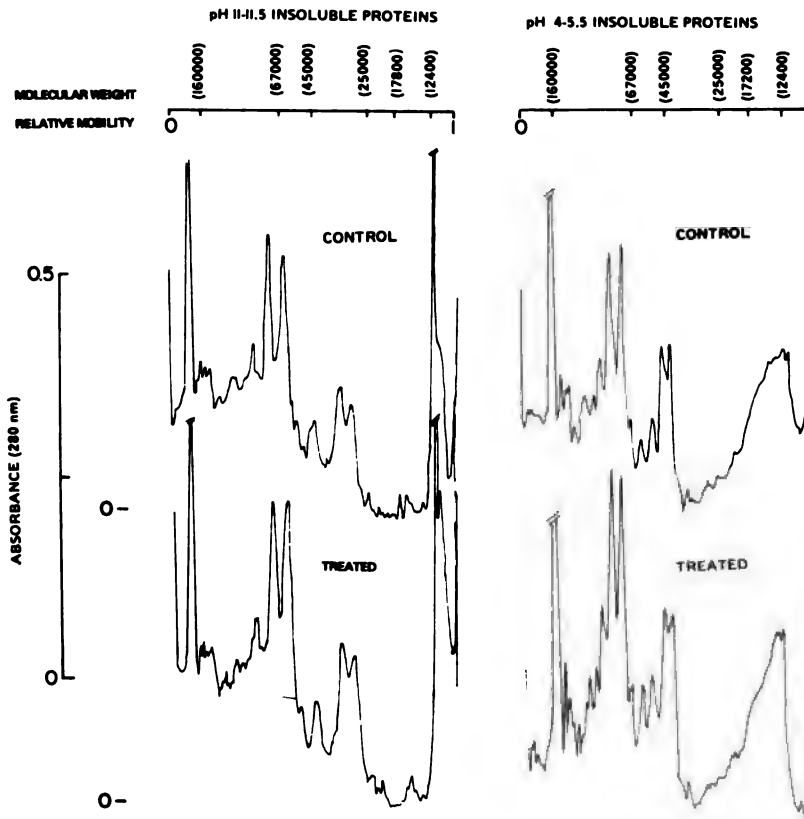
**Results and discussion.** The incorporation of [<sup>3</sup>H]leucine or <sup>3</sup>H-labeled protein hydrolysate into liver mitochondria and sub-mitochondrial fractions during cycloheximide treatment (2 mg/kg body wt) were similar to those in rat kidney (25), with an inhibition at 2 hr after stimulation at 24 hr (26). Prior to the determination of <sup>3</sup>H-labeled protein hydrolysis, radioactivity in the gel slices of the *in vitro*

tochondrial protein fractions separated by SDS-PAGE system, absorbance profiles were obtained and showed no differences between control and treated animals. A typical scan of the insoluble fractions is shown in Fig. 1.

As shown in Fig. 2, the major peaks of the activity profiles from the insoluble preparations of the control corresponded well with their respective absorbance patterns (Fig. 1). In the insoluble mitochondrial preparations (pH 11.5 and 4.5) from animals treated for 2 hr there was no inhibition of incorporation into the low molecular weight region. In contrast, during cycloheximide-stimulated synthesis (24 hr), there were of equal or, in most instances, greater incorporation than in the corresponding con-

trol fractions. As to the high molecular weight region (Fig. 2), incorporation of label into these polypeptides was significantly inhibited in the absence of cytoplasmic protein synthesis. These results obtained from *in vivo* experiments demonstrate (i) that sublethal levels of cycloheximide will transiently suppress synthesis and/or incorporation of large cytoplasmically synthesized proteins into mitochondria and (ii) synthesis and/or incorporation of this material seems to be stimulated during the recovery phase.

As to the incorporation of radioactive label into the soluble fractions (Figs. 3 and 4), labeling of high molecular weight polypeptides was inhibited at 2 hr after cycloheximide treatment and stimulated at 24 hr. Radioactivity exhibited by the materials migrated to



1. Electrophoretic distribution of insoluble polypeptides isolated from normal and cycloheximide-treated mitochondria. Isolation of mitochondria and submitochondrial protein fractions and method for SDS-polyacrylamide electrophoresis are as detailed in the text. Proteins (75  $\mu$ g) were separated at 3 mA/gel for 90 min in the anodal direction at room temperature. Molecular weight markers were:  $\gamma$ -globulin (160,000), bovine serum albumin (67,000), pepsin (45,000), chymotrypsinogen (25,000), myoglobin (17,000), and cytochrome *c* (12,400). Relative mobilities of standard proteins when plotted against  $\log(\text{molecular weight})$  gave a linear relationship. The correlation coefficient was 0.995, which was highly significant. Standard proteins were run as markers with each set of gels and a variation of molecular weight of 2000 was observed among the various runs.

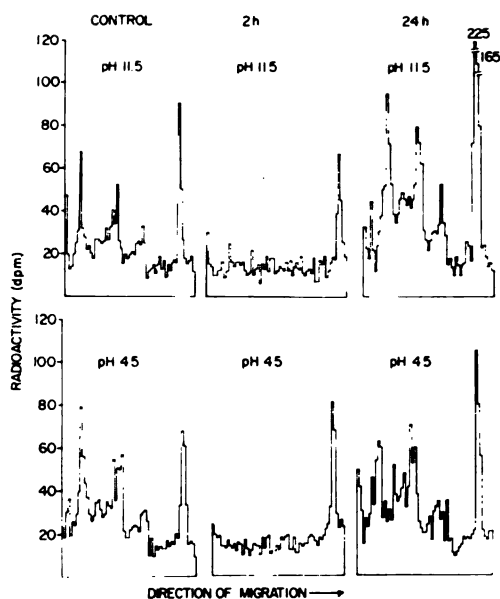


FIG. 2. Radioactivity profiles of mitochondrial insoluble proteins separated by SDS-polyacrylamide gel electrophoresis.  $^3\text{H}$ -labeled protein hydrolysate (4 mCi/kg body wt) was given 60 min prior to sacrifice. Gels were sliced into  $1.25 \pm 0.25$  mm slices by using the DE 113 horizontal gel slicer (Hoefer Scientific Instruments); each slice was solubilized in 0.5 ml of NCS solubilizer at  $50^\circ$  for 16 hr, and 10 ml of scintillation cocktail (Yorktown Research) was added before radioactivity counting. Protein (75  $\mu\text{g}$ ) was applied to the gel in each case; for other details see the legend to Fig. 1.

the front of the gel (low molecular weight region), however, showed two- to fivefold stimulation in pH 5.5, 6.5, 7.5, and 8.5 fractions during both the inhibitory and recovery phase. The labeled material in the low molecular weight range (less than 12,000) present in the aqueous extracts may represent materials other than polypeptides (i.e., aminoacyl-tRNA, or phospholipids) and there it is not possible to assess the actual contribution of mitochondrial protein synthesis to this region of the radioactivity profile.

Results presented in this paper extended the findings with *in vitro* and cultured cell systems into intact animals and suggest that high molecular weight polypeptides are either synthesized by the cytoribosomal system or the formation of functional membrane proteins requires the cooperation of both protein synthetic systems (2-12). Since in our extraction procedure lipid solvents such as chloro-

form and methanol were avoided, the molecular weight products observed in 24-hr treated animals may represent crosslinked proteolipids of the mitochondrial membrane as discussed by O'Brien (4). Furthermore, the SDS-PAGE separation of various polypeptides present in submitochondrial fractions was carried out immediately with freshly extracted samples, without age, avoiding both aggregation and degradation (4); thus, the low molecular weight materials were presumably not the result of autolysis. The cycloheximide-resistant activity appeared in the low molecular weight region of the SDS gel may suggest that the product of mitochondrial protein synthesis because gels were routinely stained with protein and scanned at 550, 280, and 260 nm and consistent patterns were obtained in all cases. However, the correlation between material and mitochondrially synthesized polypeptides requires further experimentation.

Employing lethal doses of cycloheximide *in vivo* (5, 7, 11-16), the reversal of inhibition of cytoplasmic protein synthesis and the dependence of labeled high molecular weight polypeptides can never be seen because the cytoplasmic protein synthetic system is irreversibly inhibited and the animals die within a few hours (17-20); thus, the use of high cycloheximide doses as well as the use of mitochondria in *in vitro* studies eliminate the coupling between the cytoplasmic and mitochondrial systems, thereby disallowing the effects this relationship may exert on mitochondrial translation products.

In conclusion, the interdependency of mitochondrial and cytoplasmic protein synthetic systems has been demonstrated in lower eucaryotes and cultured mammalian cells. Employing cycloheximide at a lethal dose provides a direction for an evaluation of a similar response in the living animal. There is no doubt that coordination between mitochondrion and cell sap involves an important regulatory mechanism which may be easily resolved by the *in vivo* approach. Carefully designed experimentation on whole animals may offer some insight into future investigation in the area of mitochondrial biogenesis.

**Summary.** Following *in vivo* treatment

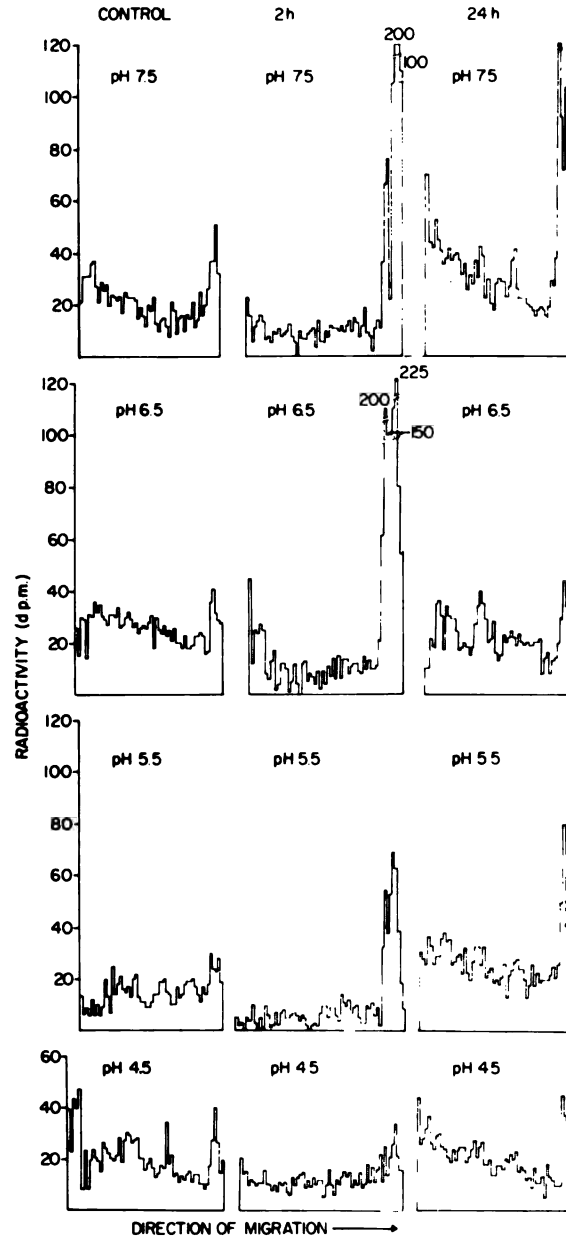


FIG. 3. Radioactivity profiles of soluble proteins extracted with acidic buffers from control and cycloheximide-treated (2 and 24 hr) mitochondria. For details see the legends to Figs. 1 and 2.

rats with a nonlethal dose of cycloheximide (2 mg/kg body wt), analysis of the newly synthesized liver mitochondrial polypeptides by SDS-PAGE system showed: (i) sublethal levels of cycloheximide did transiently suppress synthesis and/or incorporation of large cytoplasmically synthesized proteins into mi-

tochondria; (ii) synthesis and/or incorporation of this material was stimulated during the recovery phase. The differential labeling patterns of these mitochondrial proteins observed *in vivo* during cycloheximide treatment substantiate the cooperative nature of the cytoribosomal protein synthetic system to the

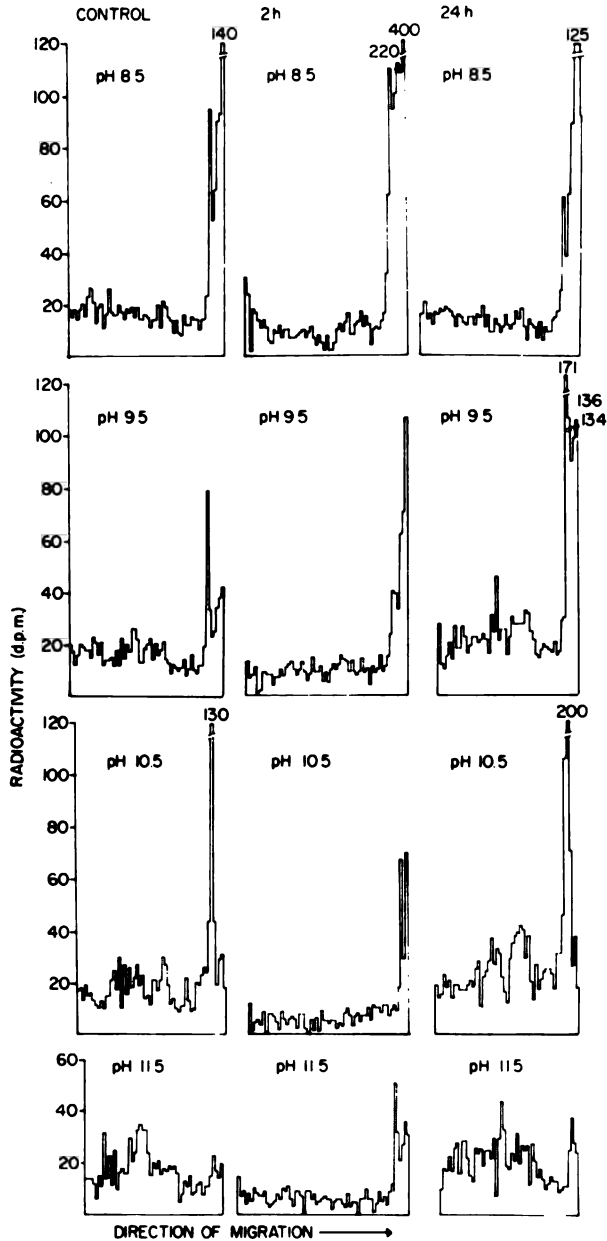


FIG. 4. Radioactivity profiles of soluble proteins extracted with alkaline buffers from control and cycloheximide treated (2 and 24 hr) mitochondria. For details see the legends to Figs. 1 and 2.

formation of functional mitochondrion observed with mammalian cells in culture.

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## Glutaminase- $\gamma$ -Glutamyltransferase: Subcellular Localization and Ammonia Production in Acidosis (40335)

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In the rat kidney, glutamine is utilized by either the mitochondrial glutaminase l-glutamate dehydrogenase pathway (1-3) or a glutaminase- $\gamma$ -glutamyltransferase pathway (4-6). The subcellular location of the glutaminase- $\gamma$ -glutamyltransferase is unknown, although if localized in the cytosol, it would lend support to the previously proposed hypothesis (7) postulating glutamine utilization by dual pathways. The present study was designed (i) to determine its subcellular location and (ii) to determine its quantitative contribution to ammonia production by acidotic kidneys perfused with 1 mM L-glutamine.

**Materials and Methods.** Male Sprague-Dawley rats, weighing between 350 and 400 g, were tube fed 1400  $\mu$ moles of  $\text{NH}_4\text{Cl}$  (0.40 M) per day for 3 days; at the end of the second day they were placed in metabolic cages, one per cage, and 24-hr urine collections were observed. Throughout the study the animals were maintained on rat chow (Purina) and water *ad libitum*. A constant ingestion of  $\text{NH}_4\text{Cl}$  ensured a similar degree of acidosis in all rats; this was confirmed by monitoring systemic blood pH and  $\text{HCO}_3$  concentration (Radiometer pH-bloodgas analyzer) at time of perfusion and determining 24-hr ammonium excretion.

Two hours prior to perfusion, rats were injected with either methionine-DL-sulfoximine (Sigma), 1.8 mmoles  $\text{kg}^{-1}$ , ip, dissolved in 1.0 ml of 0.9% saline or 0.9% saline alone. The animals were anesthetized with sodium pentobarbital, 30 mg  $\text{kg}^{-1}$ , ip, and their kidneys were isolated (8, 9) and perfused with an artificial plasma solution containing albumin (Sigma, fraction IV) and 1 mM L-glutamine; albumin was defatted (10) and dialyzed, two changes, for 48 hr against 4 liters of the perfusate solution, minus albumin. Kidneys were perfused, pH 7.40, with 80 ml of perfusate for 60 min and samples of the perfusate media were taken at 15-min

intervals. The media were analyzed for ammonia concentration by both the enzymatic (6) and the Conway microdiffusion methods, modified for blood ammonia (4); glutamine concentration was determined by measuring liberated ammonia after enzymatic (*Escherichia coli* glutaminase, Sigma) deamidation (3, 9). Ammonia production and glutamine uptake rates were calculated as described (3, 9).

Following perfusion, acidotic and acidotic plus MSO-treated rat kidneys were homogenized in ice-cold 0.44 M sucrose containing 50 mM  $\text{MgCl}_2$  and 2 mM HEPES, pH 7.4. Subcellular fractionation was carried out according to a standard schedule (11) on a Sorval RC2B refrigerated, 0-4°, centrifuge; the postmitochondrial fraction was transferred to a Beckman L ultracentrifuge and centrifuged at 105,000 g for 1 hr. The fractions obtained, nuclear + cellular debris, mitochondrial, microsomal, and soluble, were resuspended in fresh homogenizing solution and suitable aliquots were assayed for  $\text{NH}_3$  and glutamohydroxamate formation by the  $\gamma$ -glutamyltransferase reaction (5). Protein content was determined using the biuret reaction (12) employing bovine albumin (Sigma, fraction IV) as the standard.

**Results.** The response to the standard  $\text{NH}_4\text{Cl}$  load is shown in Table I. Both groups, control and pre-MSO-treated rats, received an identical acid load, exhibited a similar degree of mild acidosis, and excreted identical amounts of ammonium (coefficient of variation, 6.2 for control and 4.5 for pre-MSO-treated rats). Differences in ammonia production by perfused kidney from MSO-injected rats are not, therefore, due to a variable response to the acid load.

The effect of MSO on ammonia release and glutamine uptake is presented in Table II. Kidneys released  $50 \pm 4$  and  $48 \pm 6$   $\mu$ moles of ammonia  $\text{g}^{-1} \text{hr}^{-1}$  in the absence of exogenous glutamine. In the presence of gluta-

I. AMMONIUM CHLORIDE INTAKE, SYSTEMIC ACID-BASE BALANCE, AND AMMONIUM EXCRETION.

	Intake <sup>a</sup> NH <sub>4</sub> Cl (μmoles day <sup>-1</sup> )	Blood		Excreted NH <sub>4</sub> <sup>+</sup> (μmoles day <sup>-1</sup> )
		pH (U)	HCO <sub>3</sub> <sup>-</sup> (mEq li- ter <sup>-1</sup> )	
1	1400	7.32 <sup>c</sup> ±0.06	23.4 ±1.5	1358 ±85
2	1400	7.34 ±0.07	22.8 ±0.9	1375 ±62

<sup>a</sup> as 0.4 M NH<sub>4</sub>Cl, 1400 μmoles day<sup>-1</sup> for 3 (Methods).

administered, 0.9% NaCl, 1 ml, ip.

± SEM from four rats.

<sup>c</sup> administered, 0.9% NaCl plus MSO, 1.8

II. THE EFFECT OF MSO ADMINISTRATION ON AMMONIA RELEASE AND GLUTAMINE UPTAKE.

	Ammonia released (μmoles g <sup>-1</sup> hr <sup>-1</sup> )	Glutamine uptake (μmoles g <sup>-1</sup> hr <sup>-1</sup> )	Ammonia/ glutamine
1 <sup>b</sup>	50 ± 4	—	—
2	241 ± 24	119 ± 13	2.02
3	191 ± 18	119 ± 13	1.60 <sup>c</sup>
4	48 ± 6	—	—
5	157 ± 15	55 ± 9	2.86
6	109 ± 11 <sup>c</sup>	55 ± 9 <sup>c</sup>	1.98 <sup>c</sup>

<sup>a</sup> acidotic rats (Table I).

<sup>b</sup> of rats.

Ammonia released with 1 mM glutamine-0 mM

1.8 mmoles kg<sup>-1</sup> given 2 hr prior to perfusion. significantly different from control (*P* < 0.05).

control acidotic kidneys released 241 μmoles of ammonia while MSO-treated kidneys released significantly less (*P* < 0.01), 157 μmoles of ammonium. Glutamine uptake in acidotic controls was 119 ± 13 μmoles which fell to only 55 ± 9 μmoles (*P* < 0.05). If one assumes ammonia released, glutamine uptake accurately reflects production of the NH<sub>3</sub> produced per glutamine uptake, ratios are 2.02 ± 0.05 for acidotic kidneys and 2.85 ± 0.08 for acidotic plus glutamine-treated kidneys. Since a value of 2.0 is best possibly attained from complete deamination and deamination of glutamine, it is clear that ammonia released in the absence of glutamine contributes to the total ammonia released in the presence of glutamine. Subsequently this gives an NH<sub>3</sub>/Gln ratio of 1.60

in the acidotic control and 1.98 in the MSO-treated acidotic rats.

A direct effect of MSO on ammonia production from glutamine can be shown by adding the inhibitor to the perfusate (Fig. 1). Over the 30-min control period, the production rate averaged 1.43 μmoles min<sup>-1</sup>; within 10 min production rates fell to 1.04 μmol min<sup>-1</sup>. The fall in glutamine uptake was disproportionately greater than with ammonia production, falling from 25.9 ± 3.2 μmoles per 30 min to 16.0 ± 2.9 μmoles per min (*P* < 0.05). Consequently, the ammonia produced per glutamine extraction ratio rose from 1.66 to 1.95.

The subcellular localization of the glutamine-utilizing enzyme is shown in Table III. The activity, measured as both a glutaminase (ammonia liberated in absence of NH<sub>2</sub>OH) and γ-glutamyltransferase, appears to be a soluble enzyme for the following reasons. The activity is mainly in the soluble fraction, 56% of the total homogenate activity, and its specific activity is significantly enriched only in

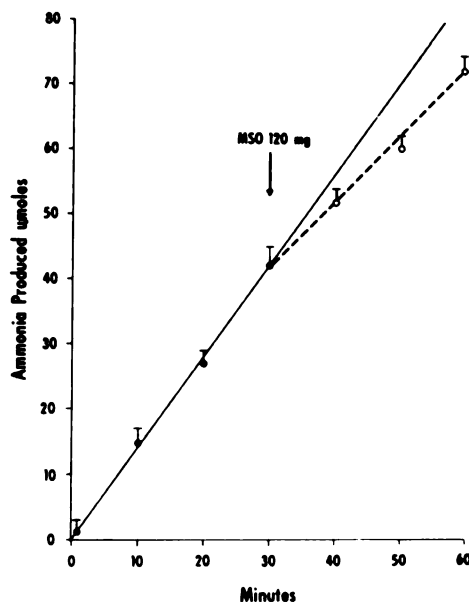


FIG. 1. Acute effect of methionine-DL-sulfoximine, 120 mg in 1.0 ml of 0.9% saline, on ammonia production from glutamine. Results are from four rats given the standard acid load (see Methods). Ammonia produced represents the total released minus the amount released in the absence of glutamine. Kidneys were perfused with 80 ml of perfusate; production rate is linear over the 60-min period in the absence of MSO.

TABLE III. RENAL GLUTAMINASE- $\gamma$ -GLUTAMYLTRANSFERASE: SUBCELLULAR LOCALIZATION AND MSO INHIBITION.

Fraction	Ammonia <sup>a</sup>		$\gamma$ -GHA <sup>b</sup>	
	Total activity <sup>c</sup>	S.A. <sup>d</sup>	Total activity	S.A.
Homogenate <sup>e</sup>				
Control	215 $\pm$ 16	1.5 $\pm$ 0.1	860 $\pm$ 45	6.0 $\pm$ 0.3
MSO	18 $\pm$ 5	0.1 $\pm$ 0.0	83 $\pm$ 8	0.6 $\pm$ 0.1
Nuclear				
Control	57 $\pm$ 8	1.8 $\pm$ 0.2	146 $\pm$ 13	4.6 $\pm$ 0.5
MSO	19 $\pm$ 11	0.6 $\pm$ 0.4	12 $\pm$ 3	0.4 $\pm$ 0.1
Mitochondrial				
Control	16 $\pm$ 5	0.3 $\pm$ 0.1	69 $\pm$ 14	1.4 $\pm$ 0.3
MSO	10 $\pm$ 7	0.2 $\pm$ 0.1	9 $\pm$ 3	0.2 $\pm$ 0.1
Microsomal				
Control	47 $\pm$ 6	2.2 $\pm$ 0.3	160 $\pm$ 21	7.4 $\pm$ 0.9
MSO	9 $\pm$ 4	0.4 $\pm$ 0.2	25 $\pm$ 12	1.0 $\pm$ 0.5
Soluble				
Control	98 $\pm$ 12	2.8 $\pm$ 0.3	481 $\pm$ 33	13.8 $\pm$ 1.0
MSO	6 $\pm$ 3	0.1 $\pm$ 0.1	19 $\pm$ 5	0.5 $\pm$ 0.1

<sup>a</sup> Ammonia produced in the absence of NH<sub>2</sub>OH.<sup>b</sup>  $\gamma$ -Glutamohydroxamate formed in the presence of NH<sub>2</sub>OH.<sup>c</sup> Total activity,  $\mu$ moles hr<sup>-1</sup>, per fraction.<sup>d</sup> Specific activity,  $\mu$ moles hr<sup>-1</sup>, per mg of protein.<sup>e</sup> Mean  $\pm$  SEM from four kidneys in each group.

the soluble fraction. Both ammonia production and  $\gamma$ -GHA formation were markedly inhibited in the soluble fraction to values less than 15% of the control. Noteworthy ammonia production by the mitochondrial fraction (glutaminase 1 pathway) was unaffected.

**Discussion.** The results clearly demonstrate the inhibition of a glutaminase- $\gamma$ -glutamyltransferase activity localized in the soluble fraction (Table III) which contributes 30 to 40% of ammonia produced by these mildly acidotic kidneys (Table II and Fig. 1). The disproportionately greater fall in glutamine uptake, 54%, than in ammonia production, 43%, is consistent with the glutaminase- $\gamma$ -glutamyltransferase pathway contributing only one ammonia per glutamine. The rise in the NH<sub>3</sub> produced/glutamine extracted ratio to 2.0 after inhibition of the cytoplasmic pathway is consistent with complete deamidation and deamination by the mitochondrial pathway. These results therefore support the previous proposal of dual glutamine-utilizing pathways in the rat kidney with NH<sub>3</sub>/Gln ratios reflective of the contribution from each pathway.

The present study underlines an important point in calculating the ammonia produced to glutamine extraction ratio (Table II). It must be realized that total ammonia release is not necessarily equivalent to that produced

from the glutamine extracted. Thus, Hems (13) observed that nonacidotic kidneys perfused with 1 mM L-glutamine released 119  $\mu$ moles of NH<sub>3</sub> per 45  $\mu$ moles of glutamine, giving an NH<sub>3</sub>/Gln ratio of 2.64; ammonia released in the absence of glutamine was similar to the present study, some 47  $\pm$  4  $\mu$ moles. Since a ratio of greater than 2 is clearly impossible, subtracting the glutamine-independent release, 47  $\mu$ moles, from 119 gives 72 actually produced from glutamine and an NH<sub>3</sub>/Gln ratio of 1.6. Ross (14) calculated an ammonia recovered to glutamine removed ratio of 1.9 with 1 mM L-glutamine as the substrate; however, if 47  $\mu$ moles of glutamine-independent ammonia release is subtracted, the ratio falls to 130 - 47 = 83/68 or 1.22; furthermore, subtracting a similar ammonia blank from the ammonia released by acidotic kidneys, 297 - 47 = 250  $\mu$ moles, and dividing by glutamine removed, 154  $\mu$ moles, gives a ratio of 1.62. In previous work, employing dextran in place of albumin, I observed an ammonia/glutamine ratio of 1.4 in nonacidotic, increasing to 1.8 in acidotic rat kidneys (3, 4, 6). Subsequently, the role of a glutaminase- $\gamma$ -glutamyltransferase was revealed in a series of studies (5, 6, 7, 15) culminating in the isolation of the enzyme from the soluble fraction (15).

The exact identity of this glutaminase- $\gamma$

yltransferase is at present unclear. It is not γ-glutamyltranspeptidase (5, 15) probably not glutamine synthetase (although this enzyme complex is capable of inase-γ-glutamyltransferase activity inhibited by MSO) since synthetase is mainly microsomal (15, 16, 17) while sent activity is predominantly soluble (13, 15). Another enzyme, γ-glutamyl-e synthetase, is a soluble protein (18) hibited by MSO (19), but does not glutamine (20). Consequently, further are required to determine the exact y of this activity.

*mary.* Glutaminase-γ-glutamyltrans- contributes some 30% of the ammonia ed from glutamine by mildly acidotic neys. The enzyme is localized in the and its inhibition results in an am- produced per glutamine extracted ra- .0. The results are therefore consistent dual glutamine-utilizing system, one ismic and the other mitochondrial, in ctioning rat kidney.

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# Accumulation of Latex in Peyer's Patches and Its Subsequent Appearance in Villi and Mesenteric Lymph Nodes<sup>1, 2</sup> (40336)

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Recent interest in the Peyer's patches of the small intestine has centered on the ability of these lymphoid structures to take in (sample) antigenic material from the intestinal lumen (1-4). Little attention has been paid to Peyer's patch uptake of inert particulates in the micron size range, in part because of the belief that large particles do not readily pass the intestinal epithelial border. We recently reported, however, that 2- $\mu$ m latex particles accumulate in Peyer's patch macrophages during chronic feeding of latex to mice (5). The present communication extends this finding and presents additional observations on the transport of particles from Peyer's patches to adjacent villi and the mesenteric lymph node.

**Materials and methods. Latex feedings.** Ten-week-old female Swiss mice (Hale-Stoner strain) were used for all experiments. Table I gives information on latex feeding to six groups of mice. A water suspension of latex (mean particle diameter  $\pm$  SD,  $2.02 \pm 0.014$   $\mu$ m; identification No. LS-1078-B, Dow Chemical Co.), was given *ad libitum* as drinking fluid. Periodic shaking of the bottles and the mixing action of air bubbles as the mice drank kept the latex suspensions relatively uniform and monodisperse. Examination of fresh intestinal contents indicated that the latex was distributed as single particles in the small intestine. All mice gained weight normally and appeared healthy.

**Tissue preparation.** To permit the examination of large amounts of tissue, clearing procedures were applied to whole Peyer's

patches and to 0.5-mm-thick slices of mesenteric lymph node. The use of xylene-based solvents, which dissolve latex, was avoided. **Peyer's patches:** Intestinal segments of ether-killed mice were fixed in 70% alcohol for several days. Peyer's patches together with small adjacent areas of intestine were excised, gently cleaned with a jet of 70% alcohol from a syringe, and rinsed in water. The tissue was treated as follows: 2% KOH, 2 hr; clearing solution I (150 ml of 2% KOH, 150 ml of glycerol, 150 ml of 0.2% formalin), 2 days; clearing solution II (100 ml of 2% KOH, 400 ml of glycerol), 2 days. Cleared Peyer's patches were stored in 100% glycerol containing a crystal of thymol. **Mesenteric lymph node:** Whole alcohol-fixed mesenteric lymph nodes were too thick and slices were too fragile for successful clearing. Slices of formalin-fixed lymph node remained intact during the clearing process but did not become as transparent as alcohol-fixed material. They were, however, sufficiently cleared by lengthening the time of exposure to 2% KOH to 2 days. Cross sections for the present study were taken from the anterior and posterior regions of the major mesenteric node.

For observation, the cleared tissue was placed on a depression slide in glycerol, coverslipped, and examined with a Zeiss inverted microscope.

**Results. Peyer's patches.** Five ileal Peyer's patches from each mouse were examined after clearing. The major structures such as crypts and villi around the patch and reticular fibers within the patch could be discerned despite the transparency of the specimens. Each patch consisted of two to eight lymphoid follicles. In mice fed high concentrations of latex (Groups A, B, D, and E), the center of each follicle on the mucosal side (the dome) was characterized by an accumulation of particles. Figure 1 shows a low-power view of such an accumulation in the dome of a Group D mouse. Under high power the par-

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<sup>2</sup> The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

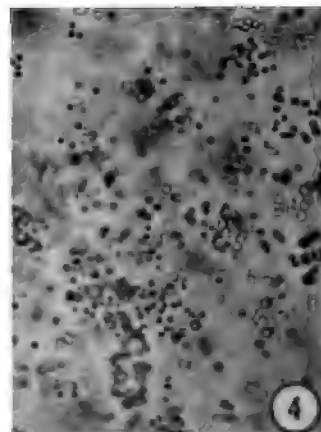
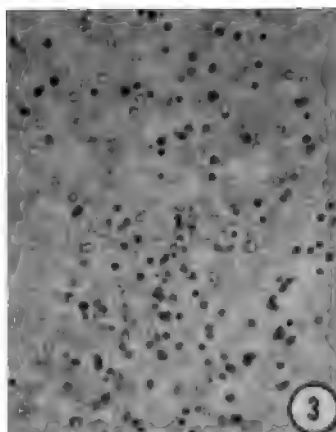
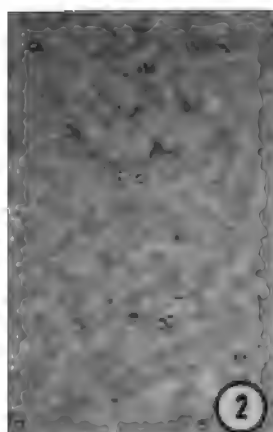
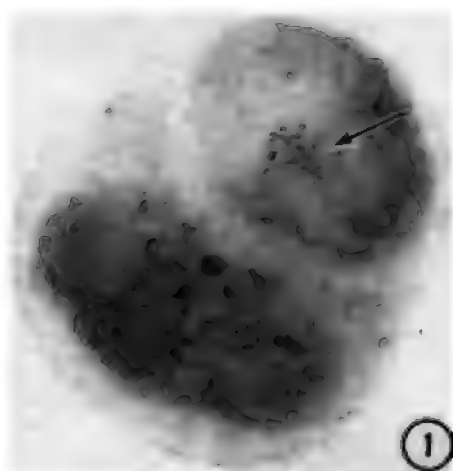
<sup>3</sup> Supported by Brookhaven National Laboratory Summer Student Program.

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TABLE I. LATEX-FEEDING REGIMENS.

	Short-term			Long-term		
	A	B	C	D	E	F
no. of animals	3	3	3	6	6	6
conc. latex (w/v)	1.0	0.1	0.01	1.0	0.1	0.01
duration (days)	3	3	3	61	61	61
washout <sup>a</sup> (days)	1.5	1.5	1.5	14-74	14-74	14-74

<sup>a</sup> Interval between termination of latex feeding and sacrifice.



1. Mucosal aspect of a cleared Peyer's patch from a young adult mouse given drinking water containing 1.0% latex for 61 days followed by 14 days without latex. Patch contains a single follicle (upper right) and two small follicles (below). Arrow points to an accumulation of latex particles in the center of the dome of the large follicle. Crypts and villi are not visible.  $\times 35$ .

2-4. Representative latex accumulations in the domes of cleared Peyer's patch follicles from mice given 1.0% (Fig. 2), 0.1% (Fig. 3), and 0.01% (Fig. 4) suspensions of 2- $\mu$ m latex as drinking fluid for 61 days. Latex feeding was terminated 14 days before sacrifice of the mice. Plane of focus is near the mucosal surface. Black circles are latex above the plane of focus.  $\times 340$ .

were refractile, uniform spheres, identical to the latex with which the mice had been fed. The amount of latex in large

patches was not uniform; central domes usually contained more latex than peripheral ones. Nevertheless, the total amount of latex

present was related to the amount fed in both short-term and long-term experiments. Figures 2 through 4 show representative latex accumulations in Peyer's patch domes. Latex could also be seen near the serosal surface of Peyer's patches from mice of Groups D and E, often in aggregates of 15 to 25 particles. Individual latex-containing macrophages could be discerned in some but not all of the specimens (Fig. 5).

In mice fed the low concentration of latex, the particles were rare (Group F) or not discernible (Group C) in Peyer's patches. Cleared Peyer's patches of control mice given tapwater to drink contained no particles that resembled 2- $\mu$ m latex although many small rod-shaped and crystalline-appearing particulates were present.

Latex particles were still present in Peyer's patches 74 days after the cessation of latex feeding in Groups D through F, but the number had declined to approximately 10% of the number seen in patches of comparably fed mice sacrificed 60 days earlier.

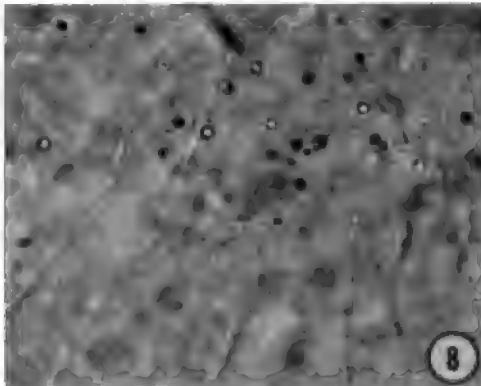
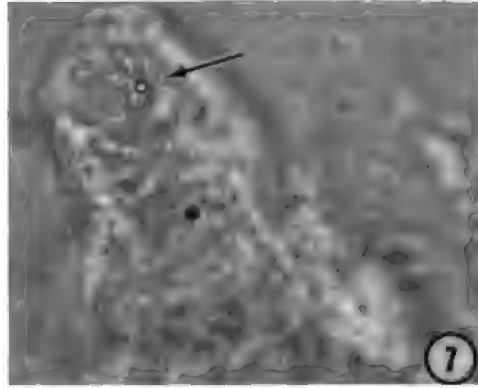
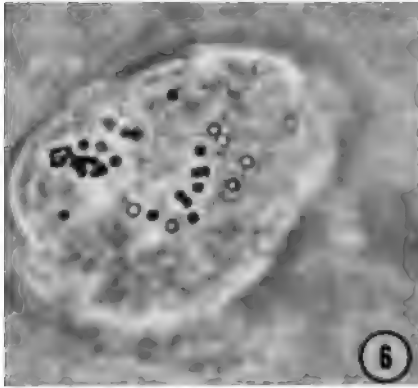
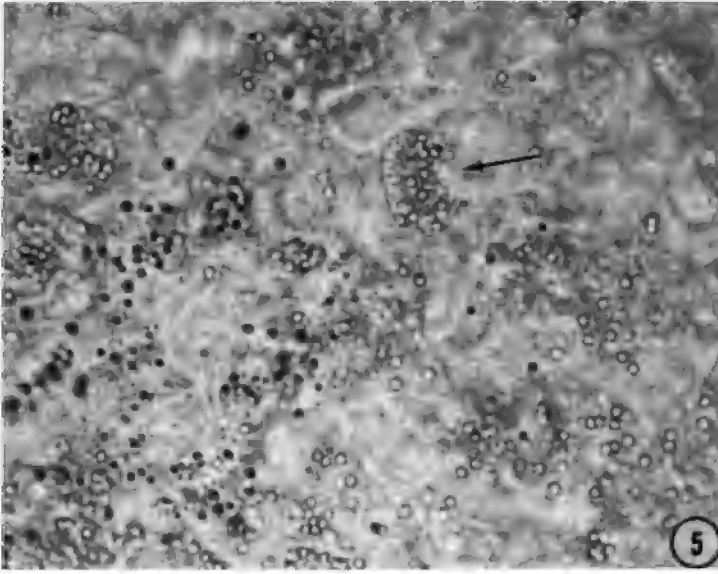
**Villi.** Latex frequently appeared in a few villi adjacent to follicles in long-term, but not short-term, experiments. In Group D mice, if a villus contained latex it almost always contained more than one particle; villi from Groups E and F usually contained only one particle. The total number of latex-containing villi was small, e.g., only 3 or 4 of the 10 to 20 villi surrounding a typical follicle might contain latex. Latex-containing villi were readily seen in a scan of the mucosal surface of a cleared Peyer's patch because of the location of particles in the villous tips (Fig. 6). The particles were usually isolated, in contrast to the close aggregates sometimes seen on the serosal side of Peyer's patches. The latex in villi was contained in granular structures, probably macrophages (Fig. 7). Latex was never observed in villi distant from Peyer's patches. No particles resembling latex were seen in villi of control mice although macrophages containing smaller particles were present. After the cessation of latex feeding, latex particles were still present in juxtafollicular villi of Groups D and E at 74 days, but in smaller numbers than at 14 days.

**Mesenteric lymph node.** Latex particles were present in cleared mesenteric lymph node tissue from latex-fed mice although they were extremely rare in short-term latex-fed

mice. All subsequent observations apply to long-term experiments. The particles were seen around germinal centers and in the central region of mesenteric lymph nodes; no particles were observed in the germinal centers themselves. Particles were more abundant in the anterior than in the posterior region of the node, and they appeared singly, never in aggregates. The total number of particles observed in lymph-node tissue was generally related to the latex concentration fed. The number of particles in mesenteric lymph-node tissue 74 days after the cessation of latex feeding was the same or larger than in comparably fed mice 14 days after the cessation of feeding. Figure 8 illustrates an area of maximum accumulation of latex in mesenteric lymph-node tissue.

**Discussion.** The present report and a previous communication from this laboratory (5) describe the accumulation and retention of 2- $\mu$ m latex particles of intestinal origin in mouse Peyer's patches. These observations support the contention that the Peyer's patch epithelium is continuously taking in (sampling) intestinal contents (1-4, 6, 7). Although latex particles remained in Peyer's patches for weeks, they were slowly eliminated after the cessation of latex feeding. The results also demonstrate the important finding that appreciable numbers of latex particles reached mesenteric lymph nodes (Fig. 8). The possibility of direct entry of latex particles into villi (8) as an explanation for the finding of latex in juxtafollicular villi (Figs. 6 and 7) cannot be totally ruled out, but our findings suggest that latex does not appear in villi until after its accumulation in Peyer's patches. The functional implications of these observations should be considered.

Peyer's patches produce immunoglobulin A (IgA) precursor cells which enter the circulation and eventually home to the mucous membranes of the gastrointestinal tract (3, 9-12). This production of IgA precursor cells is probably stimulated by the intake and transport of antigenic material through special cells in the Peyer's patch epithelium and its delivery to lymphocytes within the patch (4). The sampling of intestinal contents, however, is potentially dangerous in that it may permit entry of living pathogens and toxic materials. This risk can be minimized by delivery of sampled materials to a region rich



Portion of a cleared Peyer's patch from a mouse treated as described for Fig. 1. Macrophages (one is indicated by arrow) appear as granular bodies containing latex.  $\times 510$ .

Latex particles in the tip of a villus which adjoins a Peyer's patch from a mouse treated as described for Fig. 1.  $\times 510$ .

A latex particle (arrow) in a villus which adjoins a Peyer's patch from a mouse treated as described for Fig. 1. A latex particle is contained within a macrophage, a portion of which is visible as a stellate granular body.  $\times 510$ .

Latex particles among reticular fibers in cleared mesenteric lymph-node tissue. Mouse was given 1.0% latex in the diet for 14 days followed by 74 days without latex. Maximum accumulation of latex in this tissue is illustrated.  $\times 510$ .



in macrophages which can phagocytize and inactivate some of the toxic material; macrophages are outstandingly abundant in Peyer's patch tissue (15, 16), particularly in the immediate subepithelial zone. Thus, in overall function, the Peyer's patches may constitute a specialized system for processing intestinal antigens and particulates with little risk to the rest of the body.

Phagocytized particulate matter cannot accumulate in Peyer's patches indefinitely, and mechanisms for its elimination must be sought. Our findings suggest the existence of a population of macrophages in Peyer's patches that ingest particulate material and then migrate to neighboring villi, mesenteric lymph nodes, and possibly other locations. Since large latex aggregates were not seen in mesenteric nodes or villi, the migratory population, if it exists, has either a limited capacity to engulf particulates or a relatively short residence time in areas containing free particulates. Latex-containing macrophages that migrate from Peyer's patches to the tips of neighboring villi are probably shed into the lumen of the gut. The finding of latex in some, but not all, villi adjacent to Peyer's patches is unexplained although this may simply reflect favorable lymphatic channeling.

An alternative explanation for the finding of latex in villi and mesenteric lymph nodes after its accumulation in Peyer's patches is the movement of free particles via open lymphatic channels connected to Peyer's patches. Carter and Collins (15) have described such lymphatic connections in the mouse intestine.

The two suggested mechanisms for latex movement away from Peyer's patches (as free particles or within macrophages) are, of course, not mutually exclusive. Whether or not some particles are also shed directly from the Peyer's patch dome in the reverse of their route of entry is not known, but the finding by Bockman and Stevens (16) that the follicle-associated epithelium of appendix and Peyer's patches appears to conduct bidirectional transport of horseradish peroxidase suggests that direct elimination of particles from the dome may occur.

**Summary.** Latex particles (2  $\mu$ m in diameter) accumulated in intestinal Peyer's patches

and mesenteric lymph nodes of mice given latex suspensions as drinking fluid. After a 61-day period of latex feeding, the particles were also present in villi adjacent to Peyer's patches; they were not seen, however, after only 3 days of latex feeding. The amount of latex in Peyer's patches 74 days after the termination of latex feeding was much less than the amount present 14 days after the termination of feeding. It is suggested that migratory macrophages take up latex particles within Peyer's patches and subsequently move out of the patch to mesenteric nodes and villi. Some free particles may also be transported out of Peyer's patches to mesenteric nodes and villi through open lymphatic channels. The observations support the contention that Peyer's patches "sample" intestinal contents and they suggest a mechanism for the elimination of accumulated inert particulate matter from these lymphoid structures.

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# Evidence for Maternal and Fetal Differences in Vitamin D Metabolism (40337)

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Pregnancy induces striking changes in mineral homeostasis including the translocation of calcium and phosphorus from the mother to the fetus and elevations in the maternal levels of parathyroid hormone (1) and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) (2). These hormonal changes enhance the intestinal absorption of calcium and phosphorus by the mother and the net movement of these minerals from the maternal bone mineral to the fetus (3). The effects of these physiological alterations on the fetoplacental unit are not known at present. Furthermore, our understanding of the relationship between the mother and fetus regarding the metabolism of vitamin D and the potential interdependence in terms of the regulation of these metabolic processes is fragmentary. During the last trimester of pregnancy, maternal blood levels of 25-hydroxyvitamin D<sub>3</sub> (25OHD<sub>3</sub>) have been shown to decrease (4). A maternal to fetal gradient for the blood levels of D<sub>3</sub>, the D-metabolite produced by the liver, has been described (5). Metabolites more polar than 25OHD<sub>3</sub> were identified in the fetal homogenates after the administration of [<sup>3</sup>H]25OHD<sub>3</sub> to pregnant rats (6). In a study of similar design, differences in the maternal and fetal distribution of metabolites more polar than 25OHD<sub>3</sub> were observed but exact identity of these metabolites was not determined (7). Our studies were designed to examine the distribution and metabolism of [<sup>3</sup>H]25OHD<sub>3</sub> in selected tissues of the D-deficient pregnant rat and its changes during vitamin D supplementation.

**Materials and Methods.** Female Sprague-Dawley rats were obtained at 2 to 3 months of age and fed a synthetic, vitamin D free diet. Vitamin D deficiency was documented by the analysis of plasma 25OHD<sub>3</sub> levels by a competitive binding assay (9). After 6 weeks on this diet, the plasma levels of 25OHD<sub>3</sub> were not detectable. These rats were bred with normal males after at least 8 weeks of

the diet. The presence of spermatozoa in vaginal aspirates was used to identify the first day of pregnancy. On the 19th and 20th days of pregnancy, 0.125 μg of 25OH-[<sup>3</sup>H]26,27-D<sub>3</sub> (Amersham/Searle, specific activity 11 Ci/mmol) dissolved in 0.2 ml of ethanol was injected intravenously. On the 21st day the pregnant rats were anesthetized with ether and bled by cardiac puncture. The uterus and fetuses were exposed via a midline abdominal incision. Each fetus was removed and fetal blood was obtained by cardiac puncture. Fetal kidneys and small intestine were removed by microdissection. Plasma was separated from red blood cells by centrifugation and other tissues were minced, washed in Tris buffer (0.1 M, pH 7.4, 4°), and frozen pending homogenization. Maternal kidneys were removed, cleaned of extraneous tissue, and handled as described. Maternal small intestine was removed, cleaned with cold buffer, and opened and mucosal scrapings were obtained. Wet weight of all tissues was obtained prior to freezing and subsequent homogenization. In some animals, maternal kidneys were removed surgically under ether anesthesia prior to the first injection of [<sup>3</sup>H]25OHD<sub>3</sub>. The kidneys are currently known to be the sole organs containing the enzymes which convert 25OHD<sub>3</sub> to its two dihydroxylated metabolites, 1,25(OH)<sub>2</sub>D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> (10). Maternal nephrectomy was performed to determine if the reduction in maternal metabolites was accompanied by a parallel reduction in the fetal metabolites.

Tissues were homogenized in Tris buffer and extracted with methanol:chloroform (2:1) for 1 hr. Chloroform fractions were dried under N<sub>2</sub> and chromatographed on Sephadex LH-20 columns (2 × 30 cm) with chloroform:hexane (65:35) elution solvent. Radioactivity recovered from the LH-20 columns averaged 96% of the total extracted counts. Peak areas of radioactivity in the eluates were pooled, dried, and chromatographed on a

Spherisorb column (Laboratory Data Control, 5- $\mu$ m microsilica) using a high-pressure liquid chromatography (HPLC) system (Laboratory Data Control, Riviera Beach, Florida) for further separation and confirmation of peak identity by cochromatography with synthetic standards (25OHD<sub>3</sub>, 24R,25-(OH)<sub>2</sub>D<sub>3</sub>, and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>).

**Results and discussion.** Figure 1 depicts the LH-20 chromatograms of the maternal tissue extracts. Peaks I, II, and III cochromatographed on HPLC with 25OHD<sub>3</sub>, 24R,25-(OH)<sub>2</sub>D<sub>3</sub>, and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, respectively. The amounts of dihydroxylated metabolites formed from [<sup>3</sup>H]25OHD<sub>3</sub> are shown in Table I. The amount of each metabolite was calculated from the recovered radioactivity of the tissue extracts and the specific activity of the [<sup>3</sup>H]25OHD<sub>3</sub> given the assumption that the injected 25OHD<sub>3</sub> was the only source of vitamin D in these D-deficient animals. Based on these calculations,

maternal plasma contained 145 pg/ml of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 34 pg/ml of 24,25(OH)<sub>2</sub>D<sub>3</sub> while the maternal kidneys contained 75 pg/g wet wt of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 12 pg/g wet wt of 24,25(OH)<sub>2</sub>D<sub>3</sub>. Mucosa from the maternal small intestine contained 125 pg/g wet wt of 1,25(OH)<sub>2</sub>D<sub>3</sub> and no detectable 24,25-(OH)<sub>2</sub>D<sub>3</sub>. Fetal tissues contained different amounts and proportions of the dihydroxylated metabolites of vitamin D compared to maternal tissues. Figure 2 shows the two dominant peaks in the fetal tissues which cochromatographed on HPLC with 25OHD<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, respectively. Fetal plasma contained 40 pg/ml of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 109 pg/ml of 24,25(OH)<sub>2</sub>D<sub>3</sub>. Fetal kidneys and small intestine had no detectable 1,25(OH)<sub>2</sub>D<sub>3</sub> but contained 58 pg/g wet wt and 49 pg/g wet wt of 24,25(OH)<sub>2</sub>D<sub>3</sub>, respectively. These findings are in sharp contrast to the distribution of the metabolites in the maternal tissues (Figs. 1 and 2). Fetal plasma

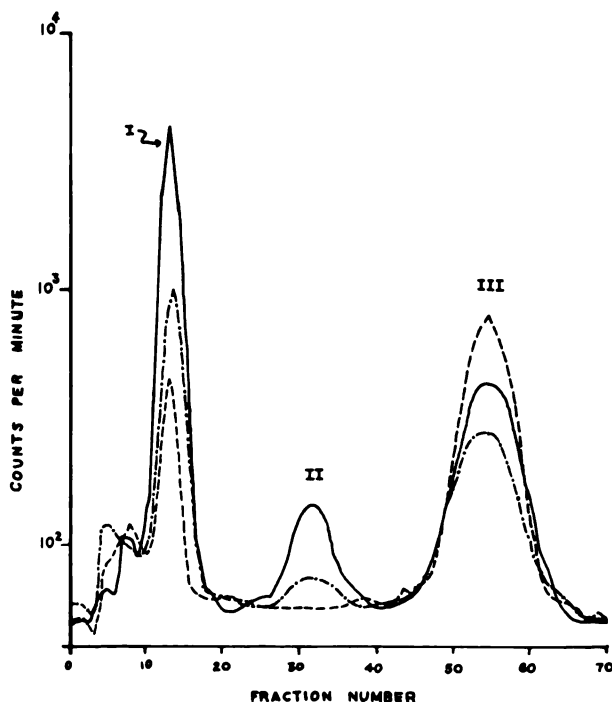


FIG. 1. Sephadex LH-20 chromatograms of maternal tissue extracts from pregnant, D-deficient rats. Maternal blood (—), kidneys (---), and small intestinal mucosal scrapings (- - -) from pregnant D-deficient rats treated with [<sup>3</sup>H]25OHD<sub>3</sub> were extracted. Dried extracts were chromatographed on LH-20 columns with chloroform:hexane solvent system. Fractions 5 ml in volume were collected. Aliquots were taken for radioactive counting on a Beckman LS-230 liquid scintillation counter (50% efficiency) in a toluene base cocktail. Remaining fraction volumes were reserved for analysis by HPLC. Radioactivity in peaks I, II, and III comigrated with synthetic 25OHD<sub>3</sub>, 24R,25(OH)<sub>2</sub>D<sub>3</sub>, and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, respectively, when analyzed by HPLC.

TABLE I. PICOGRAMS OF METABOLITES FORMED FROM [ $^3\text{H}$ ]- $250\text{HD}_3$  IN MATERNAL AND FETAL TISSUE EXTRACTS.<sup>a</sup>

	$24,25(\text{OH})_2\text{D}_3$	$1,25(\text{OH})_2\text{D}_3$	$24,25/1,25$
Maternal			
Plasma (pg/ml)	34	145	0.23
Kidneys (pg/g)	12	75	0.16
Intestinal mucosa (pg/g)	N.D. <sup>b</sup>	125	—
Fetal			
Plasma (pg/ml)	109	40	2.8
Kidneys (pg/g)	58	N.D.	—
Intestine (pg/g)	49	N.D.	—

<sup>a</sup> were calculated as picograms of metabolite based on the specific activity of the injected isotope and an 1:1 conversion of  $250\text{HD}_3$  to metabolites. Metabolite amounts were expressed either per milliliter (plasma) or per gram of wet tissue weight. Data shown are average values from three experiments.

<sup>b</sup> Not detectable.

Values are from all fetuses in each pregnant rat were pooled and results represent pooled organ content.

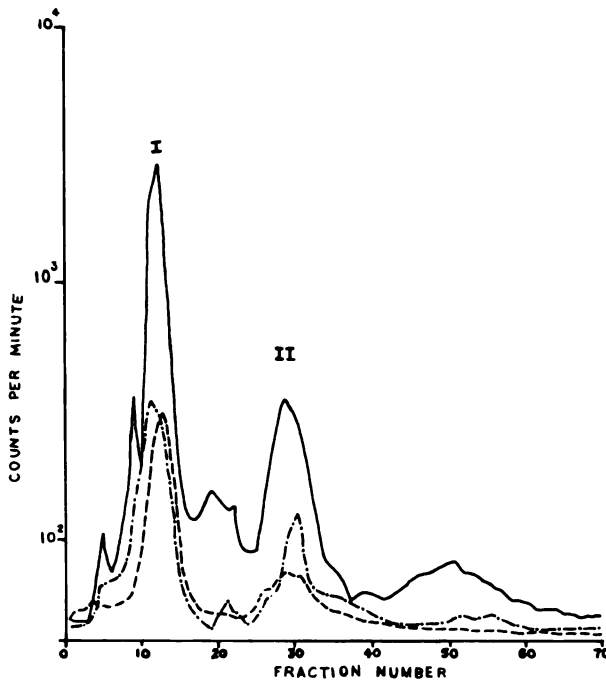


Fig. 1. Sephadex LH-20 chromatograms of fetal tissue extracts from pregnant, D-deficient mothers. Blood (—) and kidneys (— · —), and intestine (---) from fetuses of D-deficient rats treated with [ $^3\text{H}$ ] $250\text{HD}_3$  were extracted. Extracts were chromatographed on LH-20 columns with chloroform:hexane solvent system. Fractions 5 ml in volume were collected. Aliquots were taken for radioactive counting in a Beckman LS-230 liquid scintillation counter (efficiency in a toluene base cocktail). Remaining fraction volumes were reserved for analysis by HPLC. Radioactivity in peaks I and II comigrated with synthetic  $250\text{HD}_3$  and  $24R,25(\text{OH})_2\text{D}_3$ , respectively, when analyzed by HPLC.

plasma contained 320% more  $24,25(\text{OH})_2\text{D}_3$  than the corresponding maternal plasma. In contrast, fetal plasma contained 360% more  $1,25(\text{OH})_2\text{D}_3$  than the corresponding fetal plasma.

When expressed as a ratio of  $24,25(\text{OH})_2\text{D}_3:1,25(\text{OH})_2\text{D}_3$ , the ratio in maternal plasma was 0.23 and the ratio in fetal

plasma was 2.8, a 12-fold difference between the mother and the fetus (Table I).

Maternal nephrectomy (Nx) reduced the conversion of [ $^3\text{H}$ ] $250\text{HD}_3$  to its dihydroxylated metabolites. The mean plasma level of  $1,25(\text{OH})_2\text{D}_3$ , determined from three separate experiments, in the Nx mother was 36 pg/ml,

75% lower than the concentration in animals with intact kidneys. The observed difference in the maternal plasma level of  $1,25(\text{OH})_2\text{D}_3$  was highly significant ( $p < 0.001$ ).<sup>1</sup> The mean maternal plasma level of  $24,25(\text{OH})_2\text{D}_3$  was 19.5 pg/ml, a 43% reduction compared to the plasma level in animals with intact kidneys. The difference was also significant ( $p < 0.02$ ). The levels of  $1,25(\text{OH})_2\text{D}_3$  and  $24,25(\text{OH})_2\text{D}_3$  in the plasma of fetuses from these Nx mothers were 39.8 and 114.5 pg/ml, respectively. These fetal plasma levels were not significantly different from the levels observed in the fetuses from mothers with intact kidneys. When the maternal plasma levels of the dihydroxylated metabolites were lowered by Nx, the fetal plasma levels were essentially the same as the levels observed in the fetuses from mothers with intact kidneys.

The results of these studies show that the distribution and metabolism of  $[^3\text{H}]25\text{OHD}_3$  in the mother and fetus were different in blood, kidneys, and the small intestine. At a time when  $1,25(\text{OH})_2\text{D}_3$  was the dominant metabolite in maternal tissues,  $24,25(\text{OH})_2\text{D}_3$  was the dominant metabolite in fetal tissues. This difference is emphasized by the lack of detection of  $1,25(\text{OH})_2\text{D}_3$  in fetal kidneys and small intestine as well as by the 12-fold differences in the  $24,25(\text{OH})_2\text{D}_3:1,25(\text{OH})_2\text{D}_3$  ratio between maternal and fetal plasma. As expected, maternal Nx reduced the plasma levels of both dihydroxylated metabolites of vitamin D in the mother but, surprisingly, this reduction in maternal plasma levels was not associated with a parallel reduction in the fetal plasma levels. The maintenance of fetal plasma levels of  $24,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  after maternal Nx indicates that the fetoplacental metabolism of  $[^3\text{H}]25\text{OHD}_3$  is to some degree independent of the maternal metabolism. This concept of independent fetoplacental metabolism is a heretofore unsuspected aspect of vitamin D metabolism in pregnancy and fetal development. Despite these results, which demonstrate that  $24,25(\text{OH})_2\text{D}_3$  is the dominant fetal metabolite, the role of this metabolite in fetal development is unknown at present. Recent reports

describing the formation of  $24,25(\text{OH})_2\text{D}_3$  from  $25\text{OHD}_3$  in cultured chondrocytes and the stimulation of  $^{35}\text{SO}_4$  incorporation into these cells by  $24,25(\text{OH})_2\text{D}_3$  indicate that this metabolite may be involved in the growth and differentiation of the fetal skeleton (11, 12).

**Summary.** Vitamin D metabolism was studied in pregnant, D-deficient rats and their fetuses. D-depleted, pregnant rats were supplemented with  $[^3\text{H}]25\text{OHD}_3$  on the 19th day of pregnancy. The distribution and metabolism of radiolabeled D metabolites was different in maternal and fetal blood, kidneys, and small intestine.  $24,25(\text{OH})_2\text{D}_3$  was the predominant dihydroxylated D metabolite in the fetus, whereas  $1,25(\text{OH})_2\text{D}_3$  was the predominant dihydroxylated D metabolite in the mother. The ratio of  $24,25(\text{OH})_2\text{D}_3:1,25(\text{OH})_2\text{D}_3$  was 12-fold greater in fetal plasma than maternal plasma. Maternal nephrectomy reduced the metabolism of  $[^3\text{H}]25\text{OHD}_3$  to  $24,25(\text{OH})_2\text{D}_3$  (43%) and  $1,25(\text{OH})_2\text{D}_3$  (75%). However, plasma levels of these two metabolites were unchanged in the fetuses of these animals when compared with levels observed in fetuses from mothers with intact kidneys. These results suggest the possibility of independent control of  $25\text{OHD}_3$  metabolism by the fetoplacental unit and raise questions as to the possible role of  $24,25(\text{OH})_2\text{D}_3$  in fetal development.

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<sup>1</sup> Percentage differences were calculated by comparison of the mean value and  $p$  values were determined by the analysis of variance.

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# Influence of Dietary Fat, Fasting, and Acute Premature Weaning on *in Vivo* Rates of Fatty Acid Synthesis in Lactating Mice<sup>1</sup> (40338)

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Pregnancy and lactation necessitate alterations in carbohydrate and lipid metabolism to provide for fetal development and for milk production. The activities of lipogenic enzymes in rat liver and adipose tissue have been assayed to provide information on the rates of carbohydrate conversion to fatty acids in these organs during pregnancy and lactation. Reported changes in the activities of several lipogenic enzymes suggest that rates of fatty acid synthesis may be increased, decreased, or unchanged in liver (1-5) and adipose tissue (1, 5-7) of pregnant rats. Likewise, it is difficult from the reported data on lipogenic enzymes (1, 3-5, 8) to predict how lactation might alter *in vivo* rates of fatty acid synthesis in the liver. Activities of lipogenic enzymes, as measured *in vitro*, respond rather slowly to changes in flux of carbon to fatty acids; thus, it is possible that the activities of the enzymes measured did not reflect the dynamic metabolic changes which occur at the end of gestation and at the initiation of lactation.

The purpose of the present report was to evaluate the contribution of liver, adipose tissue, and mammary gland to *in vivo* fatty acid synthesis in pregnant and lactating mice; values for virgin mice were included for comparative purposes. The influence of dietary fat, fasting, and acute premature weaning on fatty acid synthesis in lactating mice was also investigated. Injection of tritiated water was utilized to obtain the *in vivo* estimates of rates of fatty acid synthesis independent of the source of the substrate (9).

**Materials and methods.** Female Swiss

Webster<sup>2</sup> mice, 10 to 12 weeks of age, were housed in solid bottom cages with wood shavings for bedding. They were fed a stock diet *ad libitum* unless indicated otherwise. Ambient temperature was  $25 \pm 2^\circ$ . Male mice were placed with female mice for 48 hr; mice which became pregnant were used in subsequent experiments. Litter size was standardized to 10 pups within 24 hr postpartum.

In one experiment virgin and lactating mice were fed one of two semipurified diets for 5 days. The high-carbohydrate diet contained, in grams per 100 g: casein, 20.0; methionine, 0.3; mineral mix (10), 4.0; vitamin mix (11), 0.4; choline chloride, 0.2; cellulose, 5.0; corn oil, 5.0; and glucose, 65.1. The high-fat diet was formulated by replacing 43.1 g of glucose with tallow on an equal energy basis. The high-carbohydrate diet contained 21, 12, and 67% energy from protein, fat, and carbohydrate, respectively, whereas the high-fat diet contained 21, 51, and 28% energy from protein, fat, and carbohydrate, respectively.

*In vivo* rates of fatty acid synthesis were calculated by determining the rate of  $^3\text{H}_2\text{O}$  incorporated into fatty acids. Each mouse was injected intraperitoneally with 0.2 ml of saline containing 1.5 mCi of  $^3\text{H}_2\text{O}$ . Mice were killed at the times indicated under results. Plasma was collected and used to obtain an estimate of the body water specific activity. All removable adipose tissue was stripped from the mice. In virgin mice, the removable adipose tissue depots comprised 55% of total body fat. Adipose tissue, liver, and mammary gland (pregnant and lactating mice) were weighed and homogenized in an equal weight of water. Aliquots were saponified and fatty acids were extracted and counted as previously described (12). Results were calculated

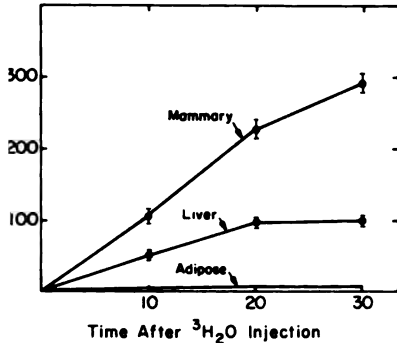
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<sup>2</sup> Spartan Research Animals, Inc., Haslett, Michigan.

<sup>3</sup> Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois.

moles of tritium incorporated into ds per organ per time interval.

s. The time sequence of  $^3\text{H}_2\text{O}$  incorporation into fatty acids in liver, adipose and mammary gland of lactating mice is shown (Fig. 1); rates of incorporation were approximately linear for the first 20 min in all three tissues. Rates of fatty acid



*In vivo* rates of fatty acid synthesis in liver, tissue, and mammary gland of lactating (3–6 d). Mice weighed  $45 \pm 1$  g; liver, adipose, and gland weights averaged  $3.0 \pm 0.1$ ,  $0.6 \pm 0.1$ ,  $0.1$  g, respectively. Each mouse was injected intraperitoneally with 1.5 mCi of  $^3\text{H}_2\text{O}$  and killed at the indicated time. Each point represents the mean  $\pm$  SEM. Values for adipose tissue were very low; therefore, individual points were deleted.

synthesis in mammary gland were approximately double the rates observed in liver. Incorporation of  $^3\text{H}_2\text{O}$  into fatty acids in adipose tissue occurred at a considerably slower rate than in mammary gland or liver. Ten minutes after injection of  $^3\text{H}_2\text{O}$ ,  $30 \pm 1$ ,  $1 \pm 1$ , and  $69 \pm 1\%$  of total fatty acid synthesis occurred in liver, adipose tissue, and mammary gland, respectively. Similar values were observed at 20 and 30 min. In subsequent experiments, mice were killed 15 min after injection of the tracer.

Body weights of 18- to 19-day pregnant mice were heavier than body weights of lactating mice; virgin mice weighed less than either pregnant or lactating mice (Table I). A marked decrease in gastrointestinal tract fill contributed to the large loss of body weight in the fasted, lactating mice. Food intake was 50% higher in pregnant mice and 147% higher in lactating mice than in virgin mice (Table I).

Liver weight increased approximately 50% in the pregnant and lactating mice and fasting for 12 hr decreased liver weight (Table I). The *in vivo* rate of fatty acid synthesis in liver, expressed per gram, was 62% lower in pregnant mice than in virgin mice; but the rates per total liver were not significantly lower

E I. *In vivo* RATES OF FATTY ACID SYNTHESIS IN LIVER, ADIPOSE TISSUE, AND MAMMARY GLAND OF VIRGIN, PREGNANT, AND LACTATING MICE.<sup>a</sup>

meter	Virgin		Pregnant		Lactating	
	Fed	Fasted	Fed	Fed	Fasted	Pups Removed
g <sup>b</sup>	$33 \pm 1^f$	$33 \pm 1^f$	$58 \pm 2^g$	$45 \pm 2^h$	$45 \pm 1^h$	$44 \pm 1^h$
body weight, g <sup>c</sup>	$+0.4 \pm 0.3^f$	$-3.2 \pm 0.3^g$	$+1.8 \pm 0.4^h$	$-0.9 \pm 0.7^i$	$-9.6 \pm 0.7^j$	$+0.6 \pm 0.9^{k,h}$
g <sup>c</sup>	$5.5 \pm 0.4^f$	$2.0 \pm 0.1^g$	$8.3 \pm 0.3^h$	$13.6 \pm 0.5^i$	$6.1 \pm 0.9^j$	$12.4 \pm 0.7^k$
min <sup>d</sup>	$1.9 \pm 0.1^f$	$1.5 \pm 0.1^g$	$2.9 \pm 0.1^h$	$3.0 \pm 0.1^h$	$2.4 \pm 0.1^i$	$3.0 \pm 0.1^h$
	$1105 \pm 256^f$	$221 \pm 12^g$	$644 \pm 97^{h,i}$	$2359 \pm 522^h$	$469 \pm 60^j$	$1964 \pm 304^h$
min <sup>d</sup>	$2.3 \pm 0.2^f$	$2.1 \pm 0.3^{g,h}$	$1.6 \pm 0.2^g$	$0.7 \pm 0.1^h$	$0.5 \pm 0.1^h$	$1.8 \pm 1.0^{f,h,k}$
	$741 \pm 194^f$	$247 \pm 41^{g,i}$	$115 \pm 37^{h,i}$	$121 \pm 26^h$	$57 \pm 21^h$	$307 \pm 66^j$
und	—	—	$2.0 \pm 0.2^f$	$3.1 \pm 0.2^g$	$2.0 \pm 0.1^f$	$4.7 \pm 0.2^h$
min <sup>d</sup>	—	—	$227 \pm 56^f$	$7059 \pm 748^g$	$392 \pm 218^f$	$2099 \pm 426^h$
m/min <sup>e</sup>	$2584 \pm 744^f$	$468 \pm 42^g$	$987 \pm 169^h$	$9540 \pm 1176^i$	$919 \pm 264^{h,k}$	$4370 \pm 707^f$
total	$60 \pm 4^{f,g}$	$50 \pm 4^{f,h}$	$66 \pm 3^g$	$24 \pm 3^h$	$62 \pm 5^{f,g}$	$48 \pm 4^h$
	$40 \pm 4^f$	$50 \pm 4^f$	$11 \pm 2^g$	$1 \pm 0.3^h$	$8 \pm 3^g$	$7 \pm 1^g$
gland	—	—	$23 \pm 2^f$	$75 \pm 3^g$	$30 \pm 6^{f,h}$	$45 \pm 5^h$

SEM for 10 mice. All mice were 10 to 12 weeks old. Mice were killed on the 18th or 19th day of pregnancy and on the 5th day of lactation. Fasted 12 hr before the fasted mice were killed and pups were removed 12 hr before one group of lactating mice was killed. Means with the same letter (f through i) are not significantly different ( $P < 0.05$ ).

<sup>a</sup> Fasted 24 hr prior to the time the mice were killed.

<sup>b</sup> Fed last 24 hr of the experiment.

<sup>c</sup> Mice were injected intraperitoneally with 0.2 ml of saline containing 1.5 mCi of  $^3\text{H}_2\text{O}$  15 min prior to killing. Values represent nanomoles of  $^3\text{H}_2\text{O}$  incorporated into fatty acids per minute per organ.

<sup>d</sup> Values obtained in liver, adipose, and mammary gland.



because pregnant mice had enlarged livers. Rates of fatty acid synthesis in livers of lactating mice were double the rates observed in virgin mice. Fasting for 12 hr, as expected, decreased tritium incorporation into hepatic fatty acids in both virgin and lactating mice. Removal of pups from the lactating mice for 12 hr did not alter rates of fatty acid synthesis in the liver.

Weight of removable adipose tissue was lower in pregnant and lactating mice than in virgin mice (Table I). Removal of pups for 12 hr resulted in a twofold increase in adipose tissue weight in lactating mice, but the increase was not significant. Rates of fatty acid synthesis were highest in adipose tissue of fed virgin mice; pregnancy, lactation, and fasting decreased tritium incorporation in fatty acids. Removal of pups for 12 hr doubled the rate of fatty acid synthesis in adipose tissue of lactating mice.

Rates of fatty acid synthesis in mammary gland were quantitated in pregnant (18–19 days) and in lactating (5 days) mice (Table I). Only minimal quantities of tritium were incorporated into mammary fatty acids in the pregnant mice, whereas the rate of fatty acid synthesis in mammary glands of the lactating mice was rapid.

The quantity of fatty acids synthesized in the three organs examined was summed

(Table I). The fed, lactating mice synthesized fatty acids at a rate nearly four times faster than observed in virgin mice and at a rate 10 times faster than observed in pregnant mice. Removal of pups for 12 hr reduced the total quantity of fatty acids synthesized by approximately 50% in lactating mice. Fasting the virgin and lactating mice markedly reduced the total quantity of fatty acids synthesized.

Approximately 50 to 60% of the fatty acid synthesis occurred in the liver of virgin mice, whereas in lactating mice only 24% of the total fatty acid synthesis occurred in the liver (Table I). The mammary gland accounted for 75% of total fatty acid synthesis in lactating mice. Adipose tissue was relatively unimportant as a site for fatty acid synthesis in pregnant and lactating mice.

The influence of a high-fat diet on fatty acid synthesis in lactating mice is presented in Table II. Values for virgin mice were included for comparison. Body weight, food intake, and liver weights were elevated in the lactating mice but adipose tissue weight was reduced relative to values observed in the virgin mice.

In agreement with the previous experiment, rates of fatty acid synthesis were elevated in the liver and depressed in the adipose tissue of the lactating mice relative to values obtained in virgin mice (Table II). High rates

TABLE II. EFFECT OF DIET ON *in vivo* RATES OF FATTY ACID SYNTHESIS IN LIVER, ADIPOSE TISSUE, AND MAMMARY GLAND OF VIRGIN AND LACTATING MICE.<sup>a</sup>

Parameter	Virgin		Lactating	
	Diet 1	Diet 2	Diet 1	Diet 2
Final body weight, g	35 ± 1 <sup>c</sup>	36 ± 1 <sup>c</sup>	43 ± 1 <sup>d</sup>	43 ± 1 <sup>d</sup>
Food intake, kcal/day	24 ± 1 <sup>c</sup>	26 ± 1 <sup>c</sup>	50 ± 3 <sup>d</sup>	56 ± 3 <sup>d</sup>
Liver				
Weight, g	2.2 ± 0.1 <sup>c</sup>	2.1 ± 0.1 <sup>c</sup>	2.9 ± 0.1 <sup>d</sup>	2.9 ± 0.1 <sup>d</sup>
FAS, nm/min <sup>b</sup>	2524 ± 364 <sup>c</sup>	605 ± 96 <sup>d</sup>	3622 ± 314 <sup>c</sup>	1017 ± 133 <sup>f</sup>
Adipose				
Weight, g	2.4 ± 0.2 <sup>c</sup>	3.2 ± 0.4 <sup>c</sup>	0.7 ± 0.1 <sup>d</sup>	0.6 ± 0.1 <sup>d</sup>
FAS, nm/min <sup>b</sup>	1204 ± 184 <sup>c</sup>	751 ± 107 <sup>d</sup>	323 ± 106 <sup>c</sup>	97 ± 28 <sup>f</sup>
Mammary gland				
Weight, g	—	—	3.9 ± 0.3 <sup>c</sup>	3.9 ± 0.2 <sup>c</sup>
FAS, nm/min <sup>b</sup>	—	—	10,200 ± 1413 <sup>c</sup>	6235 ± 1237 <sup>d</sup>
Total FAS, nm/min <sup>b</sup>	3728 ± 1086 <sup>c</sup>	1355 ± 328 <sup>d</sup>	14,145 ± 1748 <sup>c</sup>	7349 ± 1324 <sup>f</sup>
Percentage of total				
Liver	65 ± 5 <sup>c</sup>	45 ± 6 <sup>d</sup>	27 ± 3 <sup>c</sup>	16 ± 2 <sup>f</sup>
Adipose	35 ± 5 <sup>c</sup>	55 ± 6 <sup>d</sup>	2 ± 1 <sup>c</sup>	1 ± 1 <sup>c</sup>
Mammary gland	—	—	71 ± 3 <sup>c</sup>	83 ± 2 <sup>d</sup>

<sup>a</sup> Mean ± SEM for ten 10- to 12-week-old mice fed the respective diets for 5 days. Mice had been lactating for 5 days also. Means with the same superscript letter (c through f) are not significantly different ( $P < 0.05$ ). Diet 1 was a high-carbohydrate diet and Diet 2 was a high-fat diet.

<sup>b</sup> See Table I.

acid synthesis were observed in mammary gland of the lactating mice. Consumption of the high-fat diet reduced tritium incorporation into fatty acids in liver and adipose tissue of both virgin and lactating mice. Similarly, rates of fatty acid synthesis were reduced by approximately 40% in the mammary gland of lactating mice consuming the high-fat diet.

**Discussion.** The rate of fatty acid synthesis in a combination of substrates can be estimated from the incorporation of labeled hydrogen from water (9). This is a particularly useful technique to compare *in vivo* rates of fatty acid synthesis in several organs as compared to the use of a carbon tracer since the flux of carbon for fatty acid synthesis in each organ may vary (13, 14). Both liver and adipose tissue were important sites for fatty acid synthesis in the virgin mice. These results are in agreement with another report (15) and contrast with the chicken (16) where *de novo* fatty acid synthesis occurs almost exclusively in the liver and with the pig (17) where adipose tissue is the major organ for fatty acid synthesis.

*In vivo* rates of fatty acid synthesis were measured in late gestation. Even though food intake increased 50%, carbon flux to adipose tissue was reduced by more than half in pregnant mice. The energy demand for fetal development in late gestation would be expected to divert glucose from maternal utilization, thereby contributing to the lowered rate of fatty acid synthesis in the pregnant mice. Measurements of lipogenic enzyme activities in late gestation have not generally indicated such a marked reduction in fatty acid synthesis in pregnant animals. In fact, hepatic lipogenic enzyme activities in pregnant rats have often been reported to either increase (3, 4) or to increase (3, 5). These results illustrate that activities of lipogenic enzymes, as measured *in vitro* under optimal conditions, do not always reflect *in vivo* rates of fatty acid synthesis.

Mammary gland of the lactating mice exhibited an intense rate of fatty acid synthesis. Rates of fatty acid synthesis were also elevated in livers of lactating mice, but the rates of fatty acids synthesized in adipose tissue were markedly depressed. These organ-specific responses during lactation allow the lac-

tating mouse to direct dietary energy toward milk fat synthesis.

Smith *et al.* (8) have suggested that lipogenesis in the mammary gland does not respond to alterations in dietary fat, but others have obtained reduced rates of fatty acid synthesis in mammary gland preparation from rats fed high-fat diets (18) or fasted (19). Clearly, a short-term fast or consumption of a high-fat diet depressed *in vivo* rates of fatty acid synthesis in mammary gland of lactating mice. Abrupt weaning also depressed fatty acid synthesis in the mammary gland. Similarly, unilateral ligation of the teats reduced lipogenic enzyme activities in the ligated mammary gland but not in the contralateral suckled mammary gland of rats, suggesting that engorgement of the mammary gland with milk has a direct effect on the lipogenic process (20).

**Summary.** *In vivo* rates of fatty acid synthesis in liver, removable adipose tissue, and mammary gland were obtained in mice. Liver contributed 60 to 65%, and adipose tissue 35 to 40% of the fatty acids synthesized in virgin mice fed a high-carbohydrate diet. Mice in the 18th and 19th day of gestation synthesized less than half the quantity of fatty acids synthesized in virgin mice, even though the pregnant mice consumed more food than the virgin mice. Rates of fatty acid synthesis were elevated more than threefold in lactating mice and 71 to 83% of the fatty acid synthesis occurred in the mammary gland of the lactating mice. Fasting for 12 hr or consumption of a high-fat diet for 5 days depressed rates of fatty acid synthesis in all three tissues examined. Removal of the pups for 12 hr decreased the rate of fatty acid synthesis in mammary gland and increased the rate in adipose tissue of lactating mice.

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## S-Adenosylhomocysteine Metabolism in Rat Hepatomas<sup>1</sup> (40339)

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Methionine metabolism in neoplasms may differ significantly from metabolism in normal tissue (Fig. 1). Changes in the rates of synthesis of polyamines (1) and the methylation of macromolecules (2-7) indicate an increased requirement for S-adenosylmethionine. In turn, this implies a greater need for precursor methionine which could be achieved by an increase in homocysteine remethylation relative to transsulfuration (cystathionine synthesis).

In an earlier study of six rat hepatoma cell lines, we measured the tumor content of five enzymes of methionine metabolism (8). We found considerable variation between the cell lines and between the tumors and liver. However, we did not observe any changes characteristic of neoplasia. Specifically, we cannot define an enzymatic basis for the observed changes in methionine metabolism. An alternative regulatory hypothesis focuses on S-adenosylhomocysteine. This metabolite, which is the product of all transmethylation reactions which utilize S-adenosylmethionine as the methyl donor (Fig. 1, reaction 2) is hydrolyzed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1; Fig. 1, reaction 3)<sup>2</sup>—an enzyme present in virtually all mammalian tissues (10, 11). Adenosylhomocysteine possesses several interesting regulatory properties. It is a potent inhibitor of several classes of transmethylation reactions (16). Adenosylhomocysteine also inhibits betaine-homocysteine methyltransferase

(Fig. 1, reaction 7) (17) and 5-methyltetrahydrofolate-homocysteine methyltransferase (Fig. 1, reaction 8) (18)—the two enzymes which can conserve methionine. Conversely adenosylhomocysteine activates the competing cystathionine synthase reaction (Fig. 1, reaction 4) (17).

Thus, a decrease in the concentration of S-adenosylhomocysteine in neoplastic tissues could result in the metabolic alterations described in the first paragraph. However, the observation that S-adenosylhomocysteine hydrolase declines when chick embryo fibroblasts are transformed following infection with Rous sarcoma virus (19) would not be consistent with this formulation. For this reason we are reporting the results of direct assays of the adenosylhomocysteine enzyme in the six lines of rat hepatoma.

Since the hepatic content of adenosylhomocysteine hydrolase increases in animals fed a high-protein diet (10), we included studies to define whether the enzyme in hepatomas was subject to similar control. In addition, we measured the effect of the tumors both on the basal level of enzyme activity in the livers of host animals and on the regulation of the hepatic enzyme by changes in the dietary protein content.

**Materials and methods.** We studied a spectrum of transplantable hepatomas which ranged from the highly differentiated hepatoma 7787 which grew at 0.7 cm/month to the less-differentiated hepatomas 5123tc and 7777 with growth rates of 4.0 to 5.0 cm/month. The Morris hepatoma cells were inoculated into the thigh muscles of male Buffalo rats. The tumor-bearing and control animals received either a high-protein (55% casein) or low-protein diet (8% casein) for the 7 to 10 days prior to sacrifice. General Biochemicals Corporation (Chagrin Falls, Ohio) supplied the diets.

When the tumors attained a diameter of

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As indicated, this enzyme is designated S-adenosylhomocysteine hydrolase (EC 3.3.1.1) despite the fact that the thermodynamics of the reversible reaction favor synthesis of adenosylhomocysteine (9). Since we are enzyme activity in the direction of synthesis, we have chosen to use the term adenosylhomocysteine synthase when we discuss our results.



d samples of all statistical compari-

ts. The mean specific activity of *S*-adenosylhomocysteine synthase in the livers of control animals was  $425 \pm 65$  units in rats fed the low-protein diet and was  $517 \pm 70$  in the high-protein group. Although the difference in specific activities was statistically significant in five of the six individual studies, less than the twofold increase observed in an earlier study with Sprague-Dawley rats (1).

The activity of *S*-adenosylhomocysteine synthase was comparable in the livers of host and tumor-bearing rats. We found no instance in which the presence of the hepatoma affected the hepatic content of enzyme or the response to dietary protein.

Figure 1 demonstrates that extracts from tumor-bearing animals contained enzyme activity. In contrast to the liver enzyme, the specific activity of *S*-adenosylhomocysteine synthase in hepatomas did not increase significantly when the host rat ingested the high-protein diet. Indeed, the only statistically significant change induced by diet was the parallel increase in activity in hepatoma-bearing animals fed the low-protein diet. **Discussion.** The regulation of the tissue concentration of adenosylhomocysteine depends on the integrity of a metabolic sequence which includes *S*-adenosylhomocystidylase linked to enzymes with the capacity to catabolize adenosine and homocysteine. In the current study, we found that

the hydrolase was present in six rat hepatoma lines. This is consistent with our previous report that these same tumors contained five other enzymes which are components of the pathway for methionine metabolism in mammalian liver (8). However, the various hepatoma lines differed in the pattern of enzyme activities. On that basis, we suggested that hepatomas 9633, 7800, and 5123tc might be incapable of conserving methionine by means of homocysteine remethylation. Conversely, hepatomas 7787, 7794A, and 7777 were relatively deficient in cystathionine synthase and might require an exogenous supply of cyst(e)ine.

In contrast, the specific activity of *S*-adenosylhomocysteine synthase in these hepatomas was remarkably constant. When we expressed the results relative to the activities in host livers, the range was 25 to 56% in animals fed the 8% casein diet and was 12 to 28% in rats fed the 55% casein ration. These relative values are equivalent to, or greater than, the relative values obtained for the other five enzymes—with the exception of one study. In hepatoma 5123tc obtained from rats fed the low-protein diet, the relative specific activities were: methionine adenosyltransferase, 99%; 5-methyltetrahydrofolate-homocysteine methyltransferase, 139%; cystathionine synthase, 225%; and betaine-homocysteine methyltransferase, 37% (8). In this hepatoma, a value of 31% may indicate a relative deficiency of *S*-adenosylhomocysteine synthase.

Clearly the present study does not define a significant role for adenosylhomocysteine in the pathochemistry of oncogenesis. The data do not support the suggestion that a deficiency of adenosylhomocysteinase may be characteristic of neoplastic tissue (19). However, adenosylhomocysteine might be present in excess as a consequence of either augmented transmethylation or the failure to catabolize adenosine. Conversely, malignant cells may contain diminished concentrations of adenosylhomocysteine. Indeed, abnormal methylation is compatible with the release of the transmethylases from product inhibition. Obviously we require detailed studies of the adenosylhomocysteine concentration in tumors of known biological properties under controlled conditions of nutrition.

**Summary.** *S*-Adenosylhomocysteine syn-

#### I. ADENOSYLHOMOCYSTEINE SYNTHASE IN RAT HEPATOMAS.<sup>a</sup>

Hepatoma <sup>b</sup>	Specific activity (nmole/mg of protein/15 min)	
	LPD	HPD
A	$127 \pm 16^c$	$80 \pm 14^d$
	$101 \pm 19$	$123 \pm 18$
	$132 \pm 24$	$160 \pm 49$
	$207 \pm 43$	$151 \pm 30$
Tc	$93 \pm 25$	$101 \pm 10$
	$48 \pm 19$	$50 \pm 20$

<sup>a</sup> Study of a specific hepatoma line included at least 10 animals fed the low-protein diet (LPD) and the high-protein diet (HPD).

<sup>b</sup> Hepatoma lines are listed in the order of increasing rate.

<sup>c</sup>  $\pm$  SD.

<sup>d</sup> Statistical significance between diet groups:  $P < 0.05$ .

thase was present in extracts prepared from six lines of rat hepatoma. There was no apparent correlation between the specific activity of this enzyme and any of the other biological properties of the tumors. The presence of the hepatoma did not affect the activity of adenosylhomocysteine synthase in livers of host animals. Hepatic enzyme activity in both host and control rats showed an adaptive increase to an increase in dietary protein. In contrast, dietary protein failed to affect the specific activity of adenosylhomocysteine synthase in five hepatomas. Paradoxically, enzyme activity in hepatoma 7787 declined when the host rats were fed a high-protein ration.

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# Depressed Splenic T Lymphocyte Numbers and Thymocyte Migratory Patterns in Murine Malaria (40340)

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Mice infected with the malarial parasite *Plasmodium berghei* develop a fulminating anemia, a concomitant severe anemia usually succumb within several weeks (1). A number of studies (3-5) have demonstrated that a significant degree of nonspecific immunosuppression is associated with malarial infection. *In vitro* studies of the T-cell response to phytohemagglutinin (PHA) have shown transient depression in mice with resolving *P. berghei yoelii* infections and permanent depression in mice with fatal *P. berghei*-infections (6). The cause of the observed nonspecific immunosuppression is unclear, although a number of possibilities have been proposed (7-10).

Alterations in lymphoid populations during infection and recovery may account, in part, for the observed changes in immune responsiveness. Depressions in B- and T-cell populations in the thymus and lymph nodes of infected mice have been reported (10). The presence of adequate numbers of splenic T lymphocytes, although not measured in the previous studies (10), are considered critical to the immune response in view of the role of the spleen in malaria. In this regard, alterations in cellularity and compartmentalization of the spleen resulting from *P. berghei yoelii* infections have been observed (9). Contrast splenic T- and B-cell populations have been reported for adult rats with resolving *P. berghei* infections and in immature young rats in which the infection was fatal (11). It is certain, however, whether these differences between young and old rats may be attributed to an age-related altered responsiveness of T- and B-lymphocyte populations in immunologically immature and mature rats to some other factor.

Different subpopulations of lymphoid cells have been shown to migrate to different sites (12). It has been shown that thymocytes, in contrast to lymph node cells, migrate predominantly to the spleen and liver. Labeled syngeneic lymphocytes subjected to heating or freeze thawing, prior to transfer, are taken up almost exclusively by the liver (13, 14). The relative increase in hepatic uptake of radioactive label has been suggested as a sensitive index of diminished cell viability (13, 14). Furthermore, administration of antilymphocytic serum directly to recipients of labeled lymphocytes caused a reduced uptake into lymphoid tissue with a striking increase in the radioactivity recovered from the liver (15).

The following study was undertaken to quantitate the absolute numbers of T lymphocytes in the spleen during the course of virulent malaria in mature mice and to determine where normal thymocytes migrate in the diseased host.

**Materials and methods.** Twelve to fifteen-week-old BALB/c mice (Charles River Labs) were injected intraperitoneally with  $2.0 \times 10^4$  erythrocytes parasitized with *Plasmodium berghei* (NK/65 strain). At designated intervals, groups of four to six control and infected mice were monitored for circulating erythrocyte, parasitemia, and splenic T-lymphocyte levels.

Erythrocytes were counted electronically (Coulter Electronics, Inc.), and the percentage parasitemias scored from blood smears stained with Giemsa. Monocellular suspensions of dispersed spleen cells were prepared in TC medium 199 (Difco Laboratories, Inc.) containing 5% fetal calf serum, by sequential passage through 19- to 23-gauge needles (16). Counts of nucleated cells were performed by hemocytometer following red cell lysis with 3% acetic acid; total numbers of lymphocytes/spleen were determined from differen-

<sup>1</sup>Submitted in partial fulfillment of the requirements for a M. S. degree in Biological Sciences, University of Lowell.



tial smears. Lymphocytes were harvested by layering 10-ml suspensions (representing 1 spleen) on 3 ml of Ficoll/Isopaque (Litton Bionetics, Inc.) and centrifuging at 900g for 30 min at 20° (17). Interface cells, containing 80 to 90% lymphocytes were washed and labeled with  $\text{Na}^{51}\text{CrO}_4$  (50 $\mu\text{Ci}$ ) (New England Nuclear). Lymphocytes were washed several times, enumerated by hemocytometer and diluted to  $2.0 \times 10^6$  cells/ml. Mouse anti-Thy-1.2 serum (AKR, Litton Bionetics, Inc.) was employed at a dilution of 1:4 and rabbit anti-mouse lymphocytic serum (Microbiological Assoc.) at a dilution of 1:8. Serum from syngeneic donors served as the control (1:8 dilution). Guinea pig complement (Cappel Labs.) was absorbed with mouse liver and spleen cells and diluted 1:4.

The numbers of  $\theta$ -bearing cells were determined by a  $^{51}\text{Cr}$  release cytotoxic assay (18). In the assay, 0.1 ml of diluted serum (normal, anti- $\theta$ , or antilymphocyte), and 0.1 ml of  $^{51}\text{Cr}$ -labeled splenic lymphocytes ( $2 \times 10^5$  cells) from normal or infected mice were incubated in duplicate 3-ml tubes at 4° for 10 min. Following the addition of 0.1 ml of complement, tubes were reincubated for 45 min at 37° in a 7%  $\text{CO}_2$  atmosphere. Thereafter, 0.5 ml of cold TC medium was added to each tube and following centrifugation, the supernatant material was assayed in a Bio-Gamma scintillation counter (Beckman Instruments). The percentage  $\theta$ -bearing splenic lymphocytes were determined in the conventional manner.

The efficiency of normal thymocytes to seed the spleen of control and infected mice was determined as follows: Normal thymus suspensions were washed with 0.83%  $\text{NH}_4\text{Cl}$ -Tris buffer (19) and labeled by incubation with  $\text{Na}^{51}\text{CrO}_4$  (200  $\mu\text{Ci}$ ). Following several washings, suspensions were examined for viability with trypan blue and diluted to contain  $1 \times 10^8$  cells/ml with a viability of at least 90%. At designated times during infection, groups of control and parasitized mice received an intravenous inoculum of  $2 \times 10^7$  cells. Twenty-four hours later, splenic  $^{51}\text{Cr}$  was determined and expressed as a percentage of injected standard. T-cell splenic seeding was determined in additional mice receiving  $5 \times 10^5$ ,  $5 \times 10^6$ , or  $2 \times 10^7$   $^{51}\text{Cr}$ -labeled thymocytes on Day 10 of infection and assayed 24 hr later.

All data reported herein is expressed as the group mean  $\pm 1$  SE. Based on Student's *t* test,  $P < 0.05$  was considered to be a significant difference.

**Results.** *Plasmodium berghei* infection in mice is characterized by a progressive parasitemia and concomitant anemia (Fig. 1) and is fatal within several weeks. During the course of infection, the numbers of nucleated splenic cells steadily increased severalfold (Fig. 2A), which easily accounted for the elevation in numbers of total lymphocytes (Fig. 2C). In distinction, the percentage (Fig. 2D) and absolute numbers (Fig. 2E) of splenic  $\theta^+$  cells (T lymphocytes) steadily declined to approximately 20% of control levels ( $P < 0.001$ ) by Day 20. The efficiency of transplanted normal  $^{51}\text{Cr}$ -labeled thymus cells to seed into the infected spleen was significantly ( $P < 0.001$ ) reduced to approximately 45% of control levels by Day 5 and gradually declined further to 30% of control values on Day 20 (Fig. 3). The seeding efficiency was relatively constant over an inoculum range of  $5 \times 10^5$  to  $2 \times 10^7$  thymus cells, when transplanted on Day 10 of infection.

**Discussion.** *Plasmodium berghei*-infected mice succumb to the effects of high parasit-

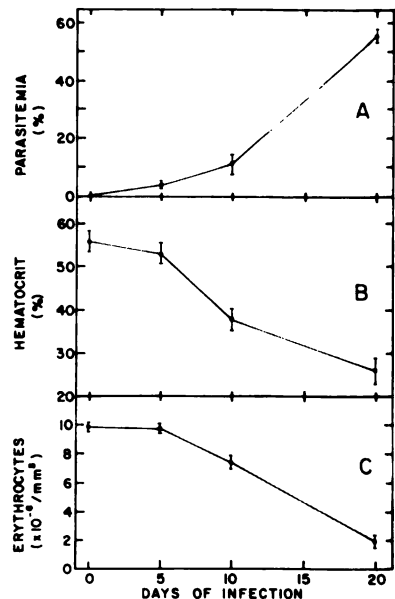


FIG. 1. Course of parasitemia and anemia following intraperitoneal injection of  $2 \times 10^4$  *P. berghei*-infected erythrocytes.

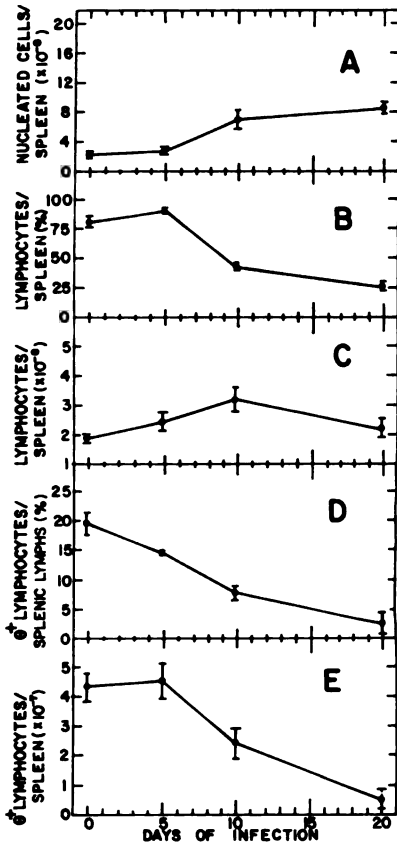


FIG. 2. Numbers of splenic nucleated cells, total lymphocytes and T lymphocytes following the intraperitoneal injection of  $2 \times 10^4$  erythrocytes parasitized with *P. berghei*.

ia and severe anemia by the third week. response to the progressive anemia (Fig. 2A). an increase in erythroid precursors (20) and macrophages (21) may have accounted part for the elevation in numbers of splenic nucleated cells in parasitized mice (Fig. 2A). The proportion of lymphocytes to total nucleated cells decreased dramatically to approximately 50% of normal on Day 10 (Fig. 2B). Nevertheless, the lymphocyte population attributed to the increased nucleated cellularity as reflected by an almost twofold rise in absolute numbers on Day 10 (Fig. 2C). In contrast to the lymphocyte population as a whole, the absolute numbers of T cells decreased on Day 10 of the infection (Fig. 2E) and the proportion of lymphocytes bearing  $\theta$  antigen was depressed to about 40% of normal controls on this day (Fig. 2D). These results compare favorably with depressions in

percentage of T cells observed in the spleens of *P. berghei*-infected young rats which succumb with high parasitemia and anemia similar to mice (11). It is possible, however, that in the latter study the absolute numbers of splenic T cells may not have been depressed, since spleen weights showed a greater than sixfold increase during the course of the infection. The decline in numbers of T cells in the spleen associated with the reported involution and depressed T-cell populations of the thymus and lymph nodes (10) suggest a general reduction in the entire pool. The observed progressive depression in T-cell numbers as well as the reported decrease in the volume of thymus-dependent areas of the spleen (9) could reflect a decline in available space as a result of the expanded erythropoietic activity (20) or could result from an overall decrease in numbers of thymus-derived cells seeding the spleen. Furthermore, adverse environmental effects in the diseased host may affect the ability of the spleen to accept T-cells or may affect the viability and/or survival of T lymphocyte populations. The thymocyte seeding study was initiated to examine this question.

The distribution patterns for thymocytes at 24 hr in normal BALB/c mice were similar to those reported for CBA mice (14). The percentage of  $^{51}\text{Cr}$ -labeled thymocytes entering the spleen markedly decreased during the course of the infection (Fig. 3). At the same time increasingly higher uptake of radioactivity was observed in the liver. Our findings that proportional distribution of thymocytes remained unchanged for the dose range ( $5 \times 10^5$  to  $2 \times 10^7$ ) in both normal (21%) and infected (8%) spleens argues against possible decreased available T-cell sites in the infected

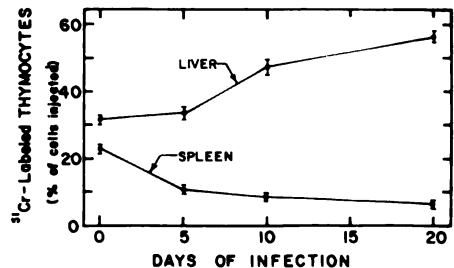


FIG. 3. Percentage of radiolabeled thymocytes in liver and spleen of infected hosts 24 hr after the intravenous transplantation of  $2 \times 10^7$  cells.

spleen, at least at these inoculum levels. Others have shown that labeled lymph node cells in normal mice do not differ in distribution characteristics at 24 hr following intravenous injection using a four log dose range ( $10^5$  to  $10^8$  cells) (12). Treatment of aliquots of labeled lymphocyte *in vitro* with cytotoxic materials including anti-lymphocyte serum and thymocytotoxic autoantibody (NZB mice) results in a dramatic increase in the uptake of radioactive label by the liver of recipients suggesting loss of cell viability (13-15, 22, 23).

Administration of anti-lymphocytes serum directly to recipients of labeled lymphocytes also caused increased liver uptake (15). Shirai *et al.* (22) state that the increase of the liver-localizing population after treatment of lymphocyte with thymocytotoxic autoantibody is consistent with the suggestions that T-cell depletion with aging of NZB mice is mediated by a continuous process of autosensitization which causes phagocytosis. It would appear possible that the viability of the thymocytes we inoculated into infected mice was adversely affected by the parasitized host environment.

**Summary.** The results obtained in this study show that a progressive depression in the splenic T-cell population occurs in *P. berghei*-infected mice and that T-cell migration is abnormal also. Since the thymus and lymph nodes involute in *P. berghei*-infected mice (10), it is likely that the total T-cell pool is depleted in the infected mouse. The decreased ability of transplanted thymus cells to seed into the infected spleen and the decreased T-cell population may indicate that infected mice have an environment hostile to T-cell viability.

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centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm <sup>3</sup>	molal (concentration)	<i>m</i>
Curie	Ci	molar (concentration)	<i>M</i>
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	<i>N</i>
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	μl	square centimeter	cm <sup>2</sup>
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**Volume 159, Number 3, December 1978**

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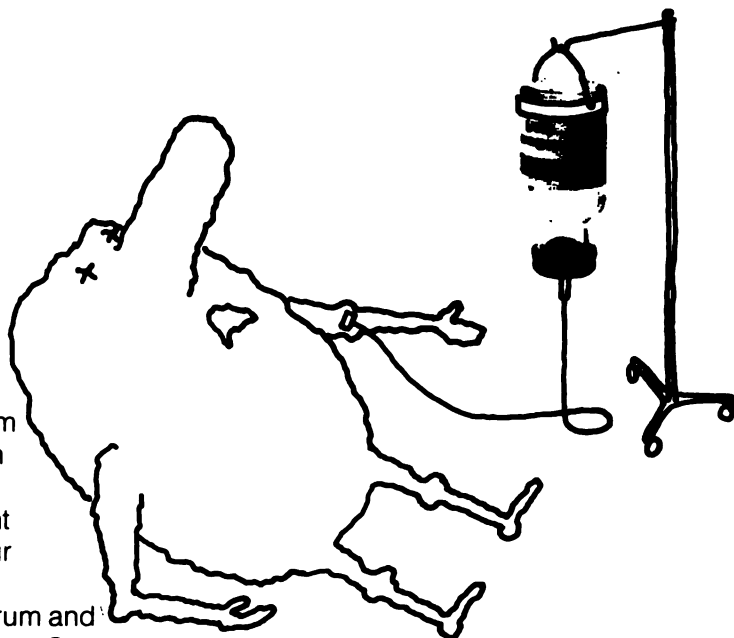
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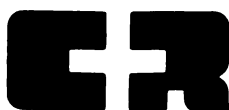
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## Direct Competition between Myoglobin and Metallothionein for Renal Reabsorption (40341)

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function of the metallothioneins (MT), metal-binding low molecular weight protein whose synthesis is induced by cadmium and other heavy metals, remains in question. Work described here was initiated in order to evaluate a possible excretory function of CdMT, and to study the mechanism of tubular reabsorption. Earlier reports provided evidence for the high capacity of kidneys to transport filtered CdMT out of the tubular lumen (1, 2). Reabsorption of CdMT proved sensitive to Cd intoxication and also be reversibly depressed by myoglobin, whereas lysozyme, immunoglobulin G and ovalbumin remained without effect. Saturability, sensitivity to inhibition and substrate specificity all support the conclusion that CdMT reabsorption represents a saturable process. The present paper explores in detail the interaction between metallothionein and myoglobin, and leads to the tentative conclusion that these two proteins compete for tubular transport by the same system. *Materials and methods.* Preparation of CdMT, and the measurement of its renal excretion characteristics and fractional reabsorption in rabbits have been described in detail in a previous publication (2). Similar techniques were applied in the study of myoglobin. Samples of horse myoglobin were purified by a standard procedure (3), and had an average final specific activity of  $10^9$  dpm/mg protein, as determined on a well-type scintillation counter (Packard Auto-γ). The protein, together with  $^3\text{H}$ -methoxyinulin, was administered as bolus injection through catheter advanced into the thoracic aorta. Radioactivity determinations included simultaneous counting of  $^{109}\text{Cd}$  and  $^3\text{H}$  in a Packard Tricarb liquid scintillation spectrometer with automatic external standardization. Alternatively, for  $^{125}\text{I}$  and  $^3\text{H}$ , total counts were determined, followed by sub-

traction of  $^{125}\text{I}$  activity as calculated from the results of  $\gamma$  counting and the ratio of  $\beta$  to  $\gamma$  counts for  $^{125}\text{I}$ . Recoveries were estimated as before (2) by summation of radioactivities of sequential urine or plasma fractions up to that fraction whose extrapolated tracer concentration fell below 2% of the cumulative total; extrapolation of the descending slope from peak values served to correct for recirculation of tracer (see e.g. Fig. 2). Mean transit time ( $\bar{t}$ ) is defined as  $\Sigma (C_i \cdot t) / \Sigma (C_i)$ , where  $C_i$  represents the concentration of tracer in a fraction collected after elapsed time  $t$  (4). The male New Zealand white rabbits used in these studies weighed on the average 2.5 kg, and had been maintained on commercial pellets. Experimental procedures were carried out under pentobarbital anesthesia. In animals with two intact kidneys diuresis was induced by continuous infusion of 5% mannitol in saline at a rate of 2 ml/kg/min. Rabbits whose left renal vein had been cannulated for measurement of A-V transit times, and whose contralateral kidney as well as mesenteric artery had been tied off, received 0.4 ml 15% mannitol in saline/kg/min.

*Results.* The low permeability of muscle capillaries to myoglobin is well documented (5), as is its relatively low glomerular sieving coefficient (6). It is not surprising, therefore, that in its artery-to-vein transit characteristics across the kidney myoglobin resembles plasma protein (Evans Blue) rather than inulin. This is illustrated in Fig. 1 by the results of one of four similar experiments; the mean transit time for Evans Blue was calculated from the ratio  $\bar{t}_{\text{EB}}/\bar{t}_{\text{In}}$  as measured in earlier work (7). In contrast, the mean vascular transit time of CdMT resembles that of inulin (2). If the rapid renal transit of myoglobin reflected primarily its binding to e.g. haptoglobulin, then it should be possible to prolong  $\bar{t}_{\text{myo}}$  by saturating and exceeding the capacity

of such ligands. However, in each of the above four studies a second bolus of myoglobin was injected, containing 1000 times the concentration used in Fig. 1 (2 mg versus 2  $\mu$ g): no significant shift in  $t_{\text{myo}}$  relative to  $t_{\text{In}}$  was observed ( $(t_{\text{myo}}/t_{\text{In}})_{\text{II}}/(t_{\text{myo}}/t_{\text{In}})_{\text{I}} = 1.03$ , range 0.89 – 1.10. In the rabbit kidney, as

in other tissues studied, free myoglobin is therefore clearly much less diffusible, and presumably less filterable, than is inulin.

It follows from the restricted diffusibility of myoglobin that the precise filtered load and, therefore, the fractional reabsorption of the protein cannot be accurately defined under present conditions. Accordingly, the following experiments on factors influencing tubular handling of myoglobin compare absolute excretion of  $^{125}\text{I}$ -myoglobin under various conditions, rather than its fractional reabsorption. Figure 2 shows the urinary transit characteristics of myoglobin at low and high concentrations. Clearly, using inulin excretion as reference point, the excretion of  $^{125}\text{I}$ -myoglobin was increased in presence of excess unlabelled myoglobin. Results of 12 similar studies are collected in Table I and show that, on the average, excretion of label rose by 43% above control values at the high myoglobin concentrations. Attention is further drawn to the fact that the same result was achieved by addition of 1.1 mg CdMT. Such a concentration of CdMT was previously shown to exert no acute toxic effect on the kidney (2); similarly, in the present study, 1.1 mg CdMT caused no inhibition of tubular PAH transport. An additional observation illustrated in Fig. 2 is the tubular transit delay of myoglobin; such a delay was consistently observed in every study and resembles that reported for CdMT (2).

Interaction between CdMT and myoglobin

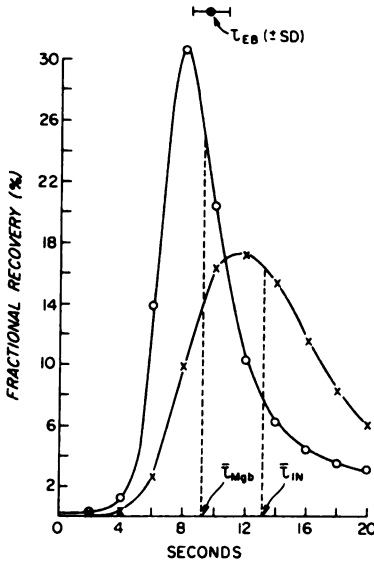


FIG. 1. A-V transit time of myoglobin. Rabbit Myo 10. Renal venous blood flow 40 ml/min, hematocrit 25%. The bolus contained 2  $\mu$ g  $^{125}\text{I}$ -myoglobin and 10  $\mu\text{Ci}$   $^3\text{H}$  inulin in a final volume of 0.3 ml. Venous recoveries are shown for  $^{125}\text{I}$  (O) and  $^3\text{H}$  (X); mean transit times ( $t$ ) are indicated, and were calculated for Evans Blue (EB) from the ratio of  $t_{\text{EB}}/t_{\text{In}}$  obtained in earlier studies (7).

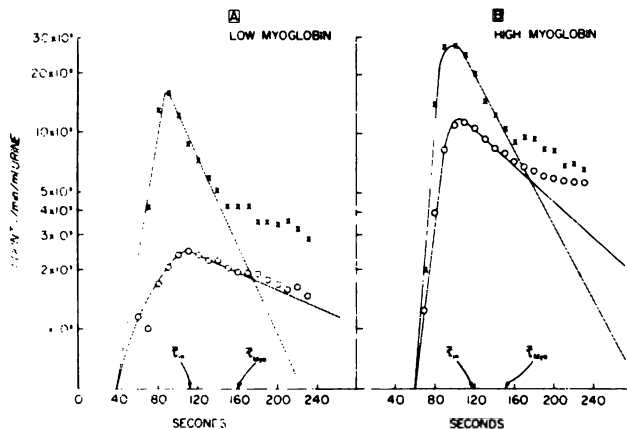


FIG. 2. Tubular transit of myoglobin. Rabbit Myo 16L. Urine flow period I: 2.6 ml/min, II: 2.8 ml/min. Urinary tracer recoveries are shown for  $^{125}\text{I}$  (O) and  $^3\text{H}$  (X). Each bolus contained (in 0.5 ml) 2  $\mu$ g  $^{125}\text{I}$ -myoglobin + 4  $\mu\text{Ci}$   $^3\text{H}$ -inulin; bolus #2 contained in addition 2 mg unlabelled myoglobin, and was injected 15 minutes after bolus I.

TABLE I. MYOGLOBIN EXCRETION.<sup>a</sup>

Periods		Excretion of <sup>125</sup> I Period II/I	
II	n	Mean	Range
I +2 mg Myo	12	1.43	0.83-2.00
I +1.1 mg CdMT	5	1.46	1.05-1.79

ch bolus contained 2  $\mu$ g <sup>125</sup>I-myoglobin. Unlabelled myoglobin and CdMT were added as shown to bolus, which was injected 15 min after bolus I. on was computed as shown in Fig. 2.

characteristic only of low molecular weight proteins. Thus, in experiments on four rabbits (8 kidneys), in which 150-300 mg myoglobin were injected intravenously 60 min before the usual arterial bolus containing CdMT, the fractional reabsorption of  $\Gamma$  fell from a mean of 57% (SD, 10.11  $\pm$  10%.

**Discussion.** Table I shows that an excess of unlabelled myoglobin increases excretion of labelled myoglobin, a result which could result either from displacement of the labelled compound from plasma ligands with subsequent increase in its filterability, or saturation of a common transport mechanism. Attention may be focused on the action of excess  $\Gamma$ : this protein does not react with high molecular weight plasma constituents under normal conditions (2). Although CdMT does, it does not compete with myoglobin for a common plasma protein ligand, it exerted the effect on <sup>125</sup>I-myoglobin reabsorption as excess myoglobin (Table I). It seems, therefore, that after injection of excess myoglobin we are dealing with saturation of reabsorption, not with increased fractional filterability. In other words, like CdMT, myoglobin appears to be reabsorbed from the tubule by a saturable process. Further, this process is inhibited by CdMT, just as our previous experiments had shown an affinity of myoglobin for the system mediating  $\Gamma$  reabsorption (2). Both proteins undergo similar tubular transit delays during competition. We also recall the ready reversibility of the myoglobin inhibition of CdMT reabsorption (2). A plausible explanation for these similarities in the renal handling of  $\Gamma$  and of myoglobin, and for the mutual inhibition of their reabsorption, invokes competition for a common reabsorptive system.

Such competition is unlikely to reflect only similarity in size of the two proteins, as hemoglobin also inhibits CdMT reabsorption; on the other hand, lysozyme and immunoglobulin L-chain did not affect CdMT transport (2). Whether the apparent competition between myoglobin and CdMT, and the saturation of their respective reabsorption, are events primarily associated with the first step in protein reabsorption at the brush border cell membrane (8) cannot be decided on the basis of the results described here.

**Summary.** Artery-to-vein and artery-to-urine transit characteristics of <sup>125</sup>I-myoglobin across the rabbit kidney were compared to those of cadmium-metallothionein (CdMT) labelled with <sup>109</sup>Cd, and their interaction during tubular reabsorption was determined. Both proteins are reabsorbed by a saturable system, mutually inhibit each other's reabsorption, and suffer similar tubular transit delays. On the basis of these results, and of the previous observation that myoglobin inhibition of CdMT reabsorption is fully reversible, we may tentatively conclude that the two proteins compete for reabsorption by a common transport system. This system also reacts with hemoglobin, indicating that its affinity for substrates is determined by factors other than purely size of the protein molecule.

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## Proteinuria and the Fragility of Normal and Diseased Glomerular Basement Membrane<sup>1</sup> (40342)

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There is a considerable body of knowledge with respect to the physical properties of interstitial collagen (1). It has been only recently, however that studies have appeared dealing with some of these properties in related basement membranes (BM). These are made up of collagen-like units bonded with sialoglycopeptides (2). The studies have been performed on two normal epithelial BM. Welling and Grantham dealt with the hydrostatic and osmotic conductance of isolated closed and perfused intact renal tubular segments and of such segments with the inner epithelial lining removed by the use of sodium desoxycholate. Tubular BM was found to be a relatively tough elastic structure (3). Likewise, Fisher and Wakely found the anterior lens capsule to be elastic and in fact at low stress values comparable to that of lightly vulcanized rubber (4). In both studies the authors found that the modulus of elasticity of BM was similar to that of interstitial collagen. Gelman and coworkers determined the melting temperature of collagen isolated from lens capsule by peptic digestion and found it to be significantly higher than that for interstitial collagen. They related this difference to the higher hydroxyproline content of BM collagen (5).

Direct measurements of the physical properties of vascular BM have not been reported. Access to vascular BM has to be at a capillary level. Only the capillaries of the renal glomerulus in their peripheral portions form free loops unencumbered by additional surrounding tissues. These loops are made up of a thick BM lined by a thin fenestrated endothelium on one surface and an epithelium with podocytic attachments on the opposite

surface. The loops are held in place by inner attachment to a delicate mesangium. The closest approximation to an isolate of glomerular basement membrane (GBM) unaltered by the harsh methods necessary to obtain it in pure form, was to cut-off the outermost portions of the tufts with their free loops from freshly obtained glomeruli and then by micromanipulative techniques to detach portions of the loops from their mesangial connections. In so doing, we were able to convert a portion of a loop into a single straight strand 60-100  $\mu$ m in length.

GBM has been compared to a thixotropic gel. It has been conjectured that in glomerular disease where there is increased permeability of the glomerular filter, this gel undergoes a physical change and becomes more sol-like. In fact Huang and coworkers (6) found that GBM from normal kidneys packed into a chromatographic column behaved like a highly cross-linked gel such as sephadex. GBM obtained from kidneys with nephrotoxic serum nephritis on the other hand appeared to be a more porous or less cross-linked gel. Somewhat similar results were obtained by Igarashi and coworkers (7).

It was of interest therefore to determine some of the physical properties of normal glomerular stands and to compare these values with those obtained from diseased glomeruli. Owing to technical limitations, the findings we are reporting deal with the effects of strain on the strands in terms of elasticity and fragility.

**Procedures and methods.** Male Lewis rats obtained from Microbiological Associates were used in all the experiments. However the GBM for the production of rabbit anti-rat GBM serum (NTS) was obtained from Holtzman rats. NTS was prepared in the manner described previously (8). In brief, male albino rabbits weighing 2.5 k were injected im with 100 mg GBM suspended in aluminum hydroxide gel. One-and-a half mil-

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s of the mixture were injected into each leg. The injections were repeated twice weekly intervals. The rabbits were bled 21 after the first injection. The serum was vated at 56° for 30 min and adsorbed times for one hour each, with washed blood cells from Holtzman rats. Normal t serum (NRS) used for controls was vated and adsorbed in the same way.

*Induction of nephrotoxic serum nephritis (N).* The rats weighed 150 g. They were ed iv with 2.0 ml NTS/100 g body wt. Control rats were injected with 2.0 ml NRS/body wt. Urinary proteins were deter- d 6 and 18 hr after injection and on a basis thereafter. Renal biopsies were . 6 and 24 hr and 10–15 days as well as ys after injection.

*Induction of aminonucleoside nephrosis (N).* The rats weighed 90–120 g. They injected SC daily for 7–10 days with 1.5 00 g body wt of the aminonucleoside of nycin as a 0.5% saline solution. Control vere injected daily with an equivalent ne of saline. Urinary protein values were ored daily. The food intake of the con- was adjusted to that of the nephrotic als on a day-by-day basis. Renal biop- ere taken 8–11, 12–15, 25–28, and 33–39 following the first injection.

*Urinary proteins.* The rats were placed in xolic cages with access to rat chow and . Urinary proteins were determined by ified biuret assay (9). Baseline values obtained prior to any experimental pro- es. The values were expressed as mg in/24 hrs/100 g body wt.

*Preparation of microprobes.* The micro- s were prepared by electrolysis of stain- eel wire, using a solution made up of parts of 3 M KCl and conc. HCl over- vith a thin layer of xylene to prevent ring. The finely tipped probes were d serially with 10% sodium bicarbonate, , absolute ethanol and xylene. The s were examined microscopically for an able degree of fineness. By bending the before electrolysis, the tips could be either curved or hooked (Fig. 1). Some tips of the probes were lightly dipped vulcanized rubber. Such rubber tipped s could be used for holding or anchor- ecimens.

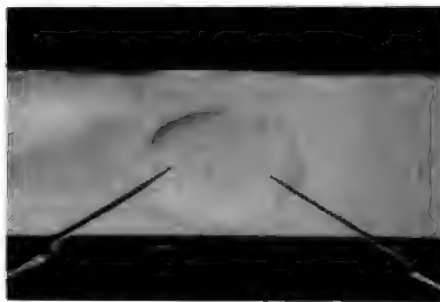


FIG. 1. Curved and hooked microprobes which were used to pluck capillary strands from the peripheral glomerular tufts.  $\times 2$ .

*Preparation of microdissection needles.* The ends of No. 11 surgical blades were attached to applicator sticks with pyseal (Fisher Scientific).

*Preparation of the renal specimens and determination of physical properties.* The renal specimens were obtained by open biopsy. They were at once placed in ice-packed tubes containing 0.15 M NaCl. They were then lightly pressed between two glass slides and rinsed with saline into a small glass dish. Glomeruli were isolated from the suspension with dissection forceps and the use of a Bausch and Lomb stereoscope provided with 25X ocular, a 0.7 to 3.0X zoom lens and a 2X auxiliary lens. The isolated glomeruli were transferred to a slide-well containing fresh saline. With microdissection needles one was able to cut off the outermost portions of the tufts of the glomeruli with their peripheral loops. These tufts with their free loops were in turn transferred to the immersion well of a slide adjusted to the stage of a Bausch and Lomb microscope. The latter was equipped with 15X ocular and Leitz 20X and 32X objectives to provide an extra working distance between the objective lens and the immersion well on the stage of the microscope. Indirect lighting was used. The microprobes were attached to two Sensaur pneumatic de Fonbrune micromanipulators. The latter were arranged in relation to the microscope so that the microprobes could be immersed and manipulated within the saline of the well of the slide. A tuft was held taut at one end with one of the probes. One of the loops of the tuft was then grasped with a curved or hooked probe. By a quick pull on the appropriate micromanipulator a portion of the loop

## FRAGILITY OF GLOMERULAR BASEMENT MEMBRANE

tached from its mesangial attachment as a single straight strand retaining the hold on the strand, stretched further either to a point it could recoil to its original length; the strain upon it or it could be the point of rupture. The strand these maneuvers remained single. Instances where more than one loop slipped away from the strand as it was being pulled. Measurements of the initial length of the strand and the extent to which it stretched beyond the initial length with an ocular micrometer which was calibrated with a stage micrometer. Pieces of rat tail tendon were cut with a microtome to suitable lengths. Thinly shaved fragments were easily attached to single strands using the microtome. Strands were treated in the same manner as the glomerular ones.

Experiments were made on glomerular strands from the experimental animals with and without MN and their appropriate controls from normal untreated rats of various ages. In each instance four or five strands from different glomeruli of the biopsied kidneys were tested. In addition we tested the effects of strain on normal

glomerular strands exposed to a variety of agents which are listed in Tables III and IV. Some of these agents were selected to simulate the biologic ones thought to be effective in producing the altered changes in the physical qualities of the glomeruli derived from the experimental and aged animals.

To verify that the alterations in the physical qualities of the glomerular strands from the experimental animals were not in vitro artifacts, the intact kidneys were perfused at normal and heightened pressures. The external surfaces of the perfused glomeruli were studied by scanning electron microscopy. The frequency of perforations as an index of increased fragility was sought for and compared with the perfused glomeruli from control kidneys.

**Renal perfusion.** Renal perfusion was performed on animals 24 hr after injection of NTS and on the 10th to the 12th day after the first injection of aminonucleoside. The animals were prepared by the iv injection of 200 units of heparin. Thirty minutes later they were perfused via the ascending thoracic aorta with 600 ml warm Ringer-Locke's solution pH 7.4, 288 mOsm/kg at 120 mm Hg. Subsequent perfusion was confined to the kidneys through the narrow sector of the abdominal aorta above and below the renal arteries. Branches of these arteries were tied as were the lumbar and spermatic arteries. The perfusion fluid was pooled human serum which had been kept chilled at all times. It was filtered through glass wool and centrifuged at 1800g. Cryoglobulins, if present were removed in a refrigerated ultracentrifuge at 30,000g. The serum prior to use was passed through a Seitz filter using a sterilizing pad with 0.4  $\mu$ m pores. Both kidneys were perfused at 120 mm Hg for 7 min. The left renal pedicle was clamped and the right kidney perfused at 300 mm Hg for an additional 7 min. By judicious clamping of the renal pedicles the pressures could be maintained in these kidneys even when followed by reperfusion with Ringer-Locke's solution and subsequently with 2.5% glutaraldehyde in Sorenson's phosphate buffer pH 7.4, 395 mOsm/kg.

**Light microscopy (LM) transmission (TEM) and scanning (SEM) electron microscopy.** Tissues were fixed in Zenker's solution for LM. Sections were stained with hematoxylin and



Portion of a microprobe (P) is seen in the lower right corner. It anchors one edge of the peripheral tuft (T). Note the several capillary loops within the tuft. There are cells in the tuft. The second microprobe (P) is seen at the top left corner. It was used to pluck the strand (S) which is stretched between it. Note that the strand has practically no cells and seems to represent glomerular basement membrane only.  $\times 560$ .

eosin, periodic acid Schiff, Masson's trichrome and Lendrum's for fibrin. For TEM the material was fixed in 4% glutaraldehyde, post fixed in buffered 2% osmium tetroxide, dehydrated and embedded in Epon. Sorval "Porter-Blum" ultramicrotomes models MT-2 and MT-2B equipped with glass or diamond knives were used for sectioning. Sections were placed on parlodion and carbon coated 75 or 200 mesh copper grids or on uncoated 300 mesh copper grids. Grids were stained with uranyl acetate and lead citrate and examined with a Hitachi HS-7S or RCA EMU-4 electron microscope. Pieces of kidney perfused with glutaraldehyde were further fixed in this solution for 2 days and then dehydrated with acetone when being prepared for SEM. The specimens were then dried in a Bomar critical point dryer with CO<sub>2</sub>. They were gold coated with a DC sputtering device to a thickness of approximately 25 Å. The specimens were examined with a Cambridge stereoscan Mark 11A scanning electron microscope at an accelerating voltage of 20 KV.

**Results.** The terms used to describe the physical properties of the glomerular capillary strands are defined as follows. Stress is a resisting force set up in the strand by the externally applied force transmitted through the micromanipulators. However, this transmitted force was so small that it could not be detected by the most sensitive gauges available to us. Strain is the change in shape that the strand underwent on applying stress. It was best expressed by the extent to which the strand could be stretched beyond its initial length. The percentage of the initial length beyond which the strand could be stretched

and still retract to its original length was regarded as a measure of its elasticity. The percentage of the initial length beyond which the strand could be stretched to the point of rupture was regarded as a measure of its fragility. Hence a normal capillary strand could for example tolerate 60% strain before rupturing, that is a strand 60  $\mu$ m in length could be stretched 36  $\mu$ m for a total length of 96  $\mu$ m. By contrast, a capillary strand from a diseased glomerulus ruptured as soon as it was stretched beyond its initial length and hence its tolerance was 0% strain. Between these two extremes one could use such terms as slight to marked increase in fragility. The elasticity and fragility of the strands could therefore be expressed in quantitative and qualitative terms without reference to the undetermined amount of stress. Valid comparisons could therefore be made between strands from normal and from diseased glomeruli.

The glomerular strands from untreated and treated control animals measured 60–100  $\mu$ m in length. They were considerably elastic. They could be stretched up to 40% beyond their initial length with good recoil. However the strands would break if stretched from 60.2% to 77.1% beyond their initial length (Tables I–IV). By contrast single strands of rat tail tendon had little elasticity and broke when stretched to 12% beyond their initial length.

The elastic properties and the degree of fragility of the glomerular strands from the experimental animals were strikingly different. It was easier to isolate the glomerular strands from the animals with NTN and AMN than from the controls. There were

TABLE I. RELATION OF FRAGILITY OF GLOMERULAR STRANDS TO PROTEINURIA IN NEPHRITIC RATS.

Days after injection	With rabbit anti-rat GBM serum		With normal rabbit serum	
	Strain <sup>a</sup>	Urinary protein <sup>b</sup>	Strain <sup>a</sup>	Urinary protein <sup>b</sup>
0.25	4.4 $\pm$ 1.1 Median 0.0	70.7 $\pm$ 9.4	66.4 $\pm$ 2.1	6.2 $\pm$ 0.9
1.0	2.2 $\pm$ 2.7 Median 0.0	55.5 $\pm$ 11.7	60.2 $\pm$ 4.4	4.3 $\pm$ 2.3
10–15	5.2 $\pm$ 6.4 Median 0.0	40.0 $\pm$ 6.2	62.0 $\pm$ 4.2	4.4 $\pm$ 0.6
60	51.4 $\pm$ 10.2	1.5 $\pm$ 1.0	68.4 $\pm$ 4.8	4.1 $\pm$ 1.1

<sup>a</sup> The percentage of the initial length beyond which a glomerular strand could be stretched before breaking. The values are the mean  $\pm$  SD of the measurements made on four or five strands from each of three rats. Medians are given for the rats treated with rabbit anti-rat GBM serum because of a skewed distribution of the values.

<sup>b</sup> Urinary protein is expressed as mg/24 hr/100 g body wt.

mucoid threads associated with the isolation of the glomerular strands most prominently from animals with NTN 6 hr after injection of NTS. Eighty-seven percent of the strands from the animals with NTN from 6 hr through the 10–15th day failed to tolerate any strain and broke immediately on stretching. Thirteen percent of the strands broke after tolerating a mean percentage strain of 32.4. The overall mean for all strands at any given time interval including those with 0% strain is given in Table I. In all instances, these changes in fragility coincided with pronounced proteinuria (Table I). In the case of the animals with AMN, all glomerular strands without exception broke immediately

on stretching. Again this coincided with proteinuria as recorded 8–11, 12–15 and 25–28 days following the initial injection of the aminonucleoside (Table II). The glomerular strands both with NTN and AMN were so fragile that it was not possible to determine their elasticity. These changes were however reversible. With the diminution of proteinuria to normal values as observed 60 days after injection of NTS (Table I) and 33–39 days after administration of the aminonucleoside (Table II), the elasticity and fragility of the glomerular strands reverted to near normal values. However prior to the return to a normal urinary protein output random tests between the 15th and 60th day for NTN and the 28th and 33rd day for AMN indicated persistence of the high degree of fragility.

The alteration in fragility of the glomerular strands was monitored by LM and EM studies of the glomeruli of the renal biopsies. At 6 hr with NTN there was some loosening of the mesangium and the deposition of electronlucent material beneath the endothelium. There was also dehiscence of the endothelium. Polymorphonuclear leukocytes and platelets were present in the capillary lumens. They were closely applied to the capillary walls. The foot-processes of the somewhat swollen visceral epithelial cells were still largely discrete. At 24 hr many of the glomerular capillary loops were thrombosed. In

TABLE II. RELATION OF FRAGILITY OF GLOMERULAR STRANDS TO PROTEINURIA IN NEPHROTIC RATS.

Days after the first injection	With aminonucleoside		With saline	
	Strain <sup>a</sup>	Urinary protein <sup>b</sup>	Strain <sup>a</sup>	Urinary protein <sup>b</sup>
8–11	0.0	20.2 ± 1.6	69.9 ± 1.2	3.3 ± 2.1
12–15	0.0	85.3 ± 4.1	66.0 ± 4.5	3.7 ± 1.1
25–28	0.0	18.5 ± 2.2	61.6 ± 0.8	2.8 ± 2.1
33–39	59.4 ± 3.2	5.3 ± 0.8	64.1 ± 5.3	3.5 ± 0.4

<sup>a</sup> The percentage of the initial length beyond which a glomerular strain could be stretched before breaking. The values are the mean ± SD of the measurements made on four or five strains for each of four rats.

<sup>b</sup> Urinary protein is expressed as mg/24 hr/100 g body wt.

TABLE III. THE FRAGILITY OF GLOMERULAR STRANDS EXPOSED TO A VARIETY OF AGENTS.

Agent <sup>a</sup>	Strain <sup>b</sup>
40% potassium iodide in buffered saline pH 7.4.	35.9 ± 1.2 (70.2 ± 2.5)
10% formalin in buffered saline pH 7.4.	5.0 ± 3.5 (69.7 ± 3.4)
0.1 mg/ml papain in EDTA pH 7.4.	7.6 ± 4.7 (72.1 ± 3.2)
0.1 mg/ml pronase in $\frac{1}{15}$ M Sorenson's phosphate buffer pH 7.4.	0.0 (76.5 ± 4.1)
0.1 mg/ml collagenase in Tris buffer pH 7.5 or in $\frac{1}{15}$ M phosphate buffer with 0.45% NaCl pH 7.4.	5.8 ± 3.6 (77.1 ± 3.7)
0.1 mg/ml Neuraminidase in Ringer-Locke with 1% bovine serum albumin pH 7.4.	25.9 ± 11.5 (66.7 ± 2.2)
0.1 mg/ml $\beta$ -N-acetyl-D-glucosaminidase with 0.1 mg/ml bovine serum albumin and 0.01 M NaCl pH 7.4.	0.0 (75.0 ± 6.0)
0.1 mg/ml poly-L-lysine in Ringer-Locke pH 7.4.	0.0 (69.4 ± 3.2)
0.1 mg/ml protamine sulfate in Ringer-Locke pH 7.4.	1.3 ± 1.3 (69.4 ± 3.2)
0.1 mg/ml poly-L-glutamic acid in Ringer-Locke pH 7.4.	62.3 ± 8.7 (69.4 ± 3.2)
0.1 mg/ml heparin in Ringer-Locke pH 7.4.	66.4 ± 7.2 (69.4 ± 3.2)

<sup>a</sup> Twice crystallized papain, collagenase with <40 caseinase units/mg and neuraminidase with <0.1% proteolytic activity were obtained from Worthington Biochemical Corp. Pronase was obtained from Calbiochem. Poly-L-lysine, poly-L-glutamic acid and protamine sulfate were obtained from Sigma.

<sup>b</sup> The percentage of the initial length beyond which a glomerular strand could be stretched before breaking. The values are the mean ± SD of the measurements made on four or five strands. The figures in parenthesis are the mean ± SD of the values obtained by treating the glomerular strands with the buffer alone adjusted to the pH of the buffer plus agent.

TABLE IV. THE FRAGILITY OF GLOMERULAR STRANDS EXPOSED TO HISTAMINE AND 5-OH TRYPTAMINE.

Agent <sup>a</sup>	μg/ml	Strain <sup>b</sup>
Histamine base in Saline pH 7.0	1	51.8 ± 2.7 (64.6 ± 2.5)
Histamine base in Ringer Locke pH 7.4	1	48.2 ± 5.7 (66.7 ± 2.1)
Histamine base in Ringer Locke pH 7.4	3	42.0 ± 3.1 (66.7 ± 2.1)
Histamine base in Ringer Locke pH 7.4	5	37.3 ± 2.2 (66.7 ± 2.1)
Histamine base in Ringer Locke pH 7.8	7	34.6 ± 1.4 (67.3 ± 6.0)
Histamine base in Ringer Locke pH 8.0	50	39.4 ± 2.7 (64.7 ± 3.1)
Histamine base in Ringer Locke pH 8.0	100	23.2 ± 1.8 (64.7 ± 3.1)
Histamine base in Ringer Locke pH 8.1	150	0.0 (66.2 ± 3.3)
Histamine base in Ringer Locke pH 8.1	200	0.0 (66.2 ± 3.3)
Histamine Acid Phosphate in Tris buffer pH 7.5	7	48.3 ± 4.1 (67.2 ± 1.7)
Histamine Acid Phosphate in Tris buffer pH 7.4	50	32.3 ± 4.4 (71.0 ± 1.9)
Histamine Acid Phosphate in Tris buffer pH 7.3	140	18.8 ± 3.3 (68.8 ± 2.1)
5-OH tryptamine Creatinine Sulfate in Tris buffer pH 7.4	7	54.3 ± 4.3 (71.0 ± 1.9)
5-OH tryptamine Creatinine Sulfate in Tris buffer pH 7.3	50	44.6 ± 2.7 (68.8 ± 2.1)
5-OH tryptamine Creatinine Sulfate in Tris buffer pH 7.3	150	36.7 ± 2.9 (68.8 ± 2.1)
5-OH tryptamine Creatinine Sulfate in Tris buffer pH 7.3	200	27.2 ± 4.4 (68.8 ± 2.1)

<sup>a</sup> The histamine base was obtained from Pfanstiehl Chemical Co., Histamine acid phosphate from Eli Lilly Co. and 5-OH tryptamine Creatinine Sulfate from Sigma.

<sup>b</sup> The same as the footnote in Table III.

patent capillaries polymorphonuclears and platelets were still present and often apposed to bared BM. Foot processes were now irregularly approximated. The mesangial and BM changes were the same as at 6 hr but better defined. There might be some swelling of the lamina densa of the BM. Subsequent periods revealed subsidence of the inflammatory reaction, regeneration of the endothelium approximated foot processes and some GBM and mesangial thickening. There was a return to a more normal appearing glomerulus at 60 days at a time when urinary protein output and the physical properties of the capillary strands had returned to near normal. With AMN the changes during the period of proteinuria consisted of total approximation of foot processes. Occasionally there was dehiscence of these and their vacuolated visceral epithelial cells. There was no more than some thickening of GBM and an increase in mesangial matrix. At no time was there evidence for an inflammatory component comparable to that seen with NTN. With reversion to a normal urinary protein output, the glomeruli assumed a more normal appearance with largely discrete foot processes and the capillary strands derived from them resumed near normal physical properties.

The glomerular strands of untreated rats were examined periodically as they aged.

These invariably gave normal values for fragility and elasticity. With beginning proteinuria at 2.5 years of age the values of strain in two survivors decreased from normal values to a mean of 40.6 and 43.8%. At this time 12–15% of the glomeruli by LM had segmental or rarely total sclerotic changes. In the ensuing 4 weeks the values of strain dropped to a mean of 15.3%. There was a distinct decrease in elasticity. The glomerular strands failed to return to their original length or did not recoil at all, when stretched below their breaking point. Thirty percent of the glomeruli now showed marked sclerosis. These latter when isolated had yellow patches presumably representing the sclerotic loops. Strands from these loops were highly fragile and broke immediately. Less involved glomeruli had GBM and mesangial thickening and by EM fusion of foot processes over thickened loops.

The increased fragility of the diseased glomerular capillaries could be corroborated by perfusion of the kidneys and the examination of their glomeruli by SEM. Ninety-three glomeruli from 9 control kidneys perfused at 120 or 300 mm Hg showed no perforations except in one kidney perfused at 300 mm where three out of ten presented single perforations. In the case of NTN of 43 glomeruli from four kidneys perfused at 120 mm 13 or 30% showed one or more perforations. Of 63 glo-

meruli from five kidneys perfused at 300 mm 19 or 30% showed one or more perforations. With AMN of 33 glomeruli from three kidneys perfused at 120 mm 19 or 57% showed one or more perforations. At 300 mm Hg of 45 glomeruli from four kidneys 32 or 71% showed perforations (Figs. 3-5).

The effects of a variety of agents on glomerular strands isolated from normal glomeruli are presented in Tables III and IV. It is to be emphasized that these results represent changes in fragility which occurred at room temperature and often within minutes following exposure to the agent. The buffers used as controls were adjusted to the same pH as the buffer plus the agent. Forty percent KI which causes chemical contraction of collagen comparable to heat contraction increased the fragility of the glomerular strands. There is even a greater increase in fragility following fixation of the strands in 10% formalin. The proteolytic enzymes, papain, pronase and collagenase all sharply and markedly increased fragility. Neuraminidase and  $\beta$ -N-acetyl-D-glucosaminidase both increased fragility. Neuraminic acid and N-acetyl glu-

cosamine are components of the noncollagenous glycopeptides of GBM. However the striking effects of the glucosaminidase might have been due to a possible minor contaminant with a proteolytic enzyme. It is of extreme interest that by contrast with the polyanions, poly-L-glutamic acid and heparin which had no effect on the glomerular strands, the polycations, poly-L-lysine and protamine sulfate produced marked increases in fragility. Histamine and 5-OH-tryptamine both highly vasoactive substances, likewise increased the fragility of the glomerular strands. The increase was a graded one commensurate with the increasing concentration of the agent.

*Discussion.* Of the components of the wall of the glomerular capillary it is the BM which forms the structural backbone and serves as its support. In fact Murphy and Johnson (10) have submitted the data of Welling and Grantham (3) on renal tubular BM to mathematical analysis and have drawn the inference that capillary BM and particularly GBM are responsible for the rigidity and self support of these vessels. The results obtained

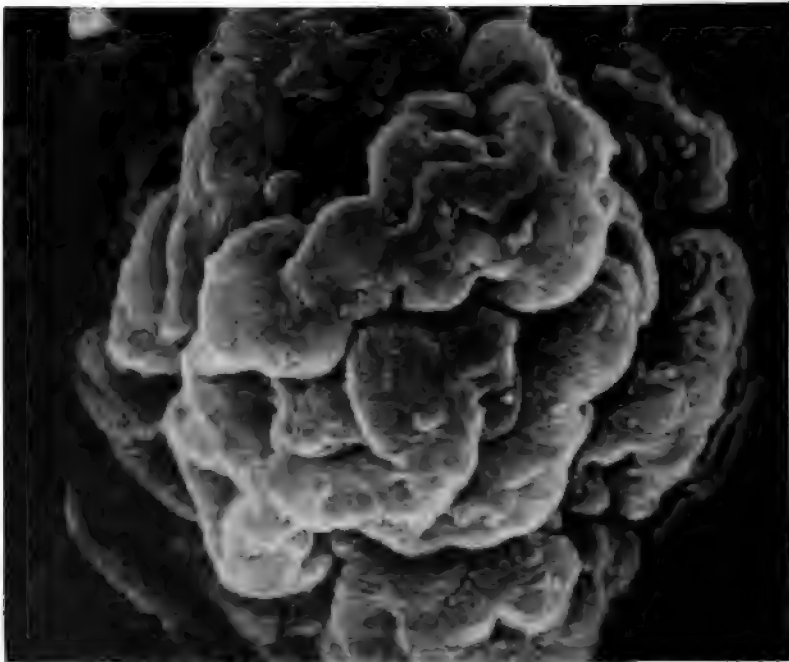


FIG. 3. Scanning electronmicroscopic view of a glomerulus from a normal kidney perfused with serum at 300 mm Hg. Details of the visceral epithelial cells and their processes can readily be made out. There are scattered microvilli. There are no perforations.  $\times 1000$ .

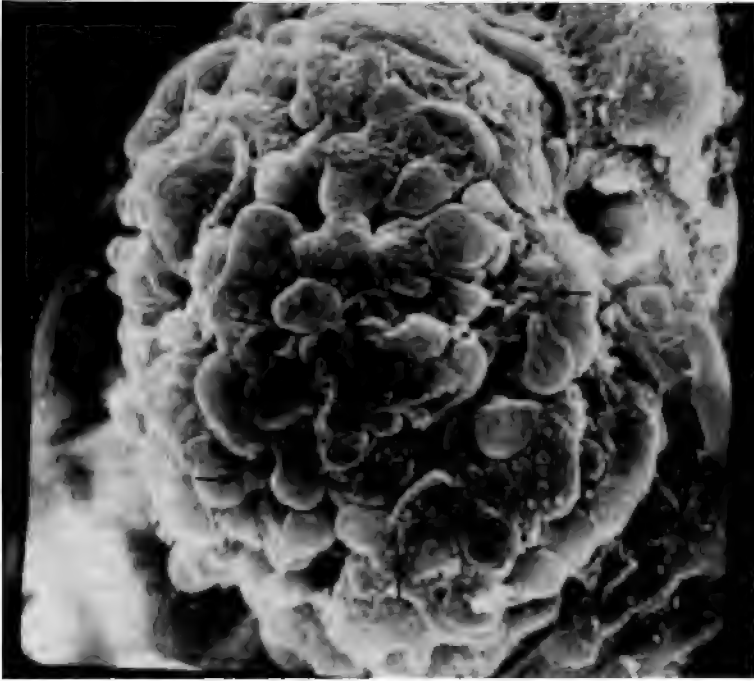


FIG. 4. Scanning electronmicroscopic view of a glomerulus from a kidney with aminonucleoside nephrosis infused with serum at 300 mm Hg. The loops are irregular and distorted. Cells and their processes are largely obliterated. The surfaces tend to be smooth with some microvilli and some blebs. There are multiple perforations indicated by the arrow head and arrows.  $\times 1000$ .

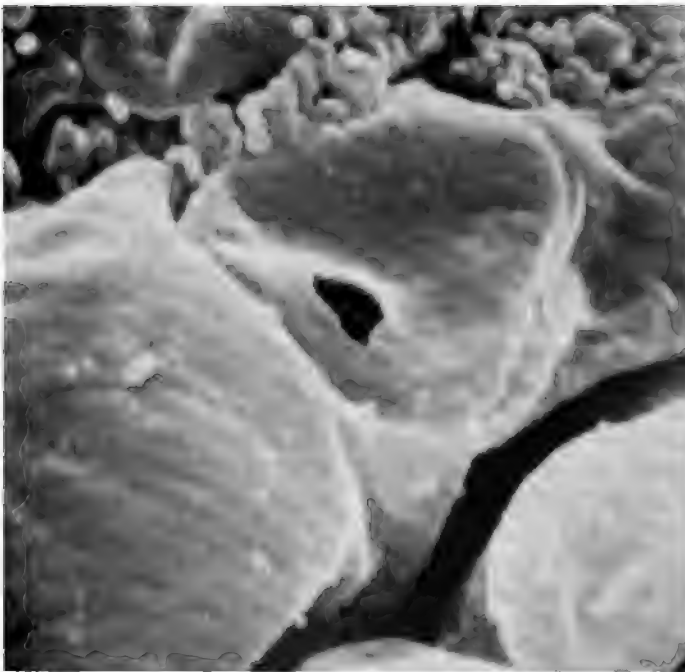


FIG. 5. As in Fig. 4. The perforation indicated by the arrow head in Fig. 4 is shown in an enlargement of the  $\times 10,000$ .



with the isolated single strands from peripheral glomerular capillaries can therefore be considered as representative of the physical properties of GBM.

Normal glomerular strands are very elastic and are resistant to rupture when stressed not unlike that reported for renal tubular BM (3) and anterior lens capsule (4). By contrast there is a marked increase in the fragility of the strands from the glomeruli of rats with NTN and AMN and from the affected glomeruli in spontaneous glomerulosclerosis. The degree of increase in fragility was such that elasticity could no longer be measured since the strands broke immediately on being stretched beyond their initial lengths. The strands from very aged rats were less elastic than normal. The changes in fragility occurred in all instances simultaneously with the onset and persistence of proteinuria. They reverted however to near normal during the recovery phase of NTN and AMN with the resumption of a normal output of urinary protein. Comparable degrees of increased fragility were observed *in vitro* by brief exposure of normal glomerular strands to proteolytic and collagenolytic enzymes, to neuraminidase and to polycations. Polyanions by contrast were without effect as were the various buffers that were used as vehicles for all these agents.

The degree of increase in fragility of the glomerular strands in NTN appeared to be somewhat variable. Not all strands broke immediately on stretching, suggesting that the damage to the capillary wall was not uniform and that the immune inflammatory response was more intense in focal glomeruli and in segmental sectors. This seemed to be borne out by the perfusion studies where a fixed 30% of the glomeruli showed perforations independent of the pressure employed whether at 120 or 300 mm Hg. The damage to GBM is assumed to occur through the release of lysosomal enzymes particularly from the polymorphonuclear neutrophils of the inflammatory exudate (11). As indicated in the *in vitro* experiments even brief exposure of the glomerular capillary strands to somewhat similar enzymes could bring about a sharp increase in their fragility. In addition to enzymatic action there is a change in the staining pattern for the anionically charged

sialoglycoproteins of the glomerular capillary wall shortly after the onset of NTN followed by a quantitative decrease in sialic acid (12). Considerable emphasis has been placed recently on the reduction of the normal anionic charge of the glomerular capillary wall with reference to increased permeability of anionically charged serum proteins such as albumin (13). In fact perfusion of the kidney with polycations can lead to proteinuria (14) and as shown here exposure of normal glomerular strands to polycations can increase their fragility promptly and markedly. In effect therefore both reduction in net negative charge and enzymatic action appear to account for the increased permeability and fragility of the glomerular capillary wall in NTN.

The increased fragility of the glomerular strands with AMN was more uniform. All strands broke immediately on being stretched beyond their initial length. Also the number of glomeruli with ruptures following perfusion was greater than with NTN and increased from 57% at 120 mm Hg to 71% at 300 mm. There is no significant inflammatory component with AMN. However, there is loss of net negative charge associated with decrease in sialic acid (15). There is also a change in the composition of the GBM with a decrease in hydroxylysine and hydroxyproline, a corresponding increase in lysine and proline and an altered glucose-galactose-hydroxylysine ratio of 2:1:1 as compared with 1:1:1 for normal GBM (16). Altered synthesis of GBM as well as reduction in negative charge may be the basis for increased permeability and fragility in AMN.

Morphologic changes in GBM with NTN and AMN have been said to vary from none to some edematous swelling in the earlier stages and to some thickening in the later stages. This is borne out by our own observations. It is not clear to what extent such changes in and of themselves contributed to increased permeability and fragility. However with spontaneous glomerulosclerosis there is variable and in the most affected loops marked GBM thickening. There are no data to indicate whether such thickened BM are associated with loss of net negative charge or with distinctive changes in chemical composition. It is known that with aging there is increased hydroxylation of lysine of the GBM

ased glycosylation of hydroxylysine  
ere is also a decrease in sialic acid  
netheless morphologically altered  
glomerulosclerosis is associated with  
l fragility and presumably with in-  
ermeability.

ld appear therefore that besides di-  
ymatic action, alterations in the  
composition and molecular config-  
of GBM including a reduction in its  
tive charge can bring about striking  
in its fragility. These changes appear  
timately associated with increased  
ility to plasma proteins. With pro-  
there is commonly approximation or  
of foot processes with displacement  
t diaphragms. This is almost univer-  
AMN, more irregular with NTN and  
er thickened loops in glomeruloscle-  
has been suggested by Seiler and  
s (19, 20) that the mobility of the  
cesses may be primarily dependent  
ered charge relationships between  
al foot processes and between foot  
and BM. With the movement of the  
esses the slit diaphragms would then  
ced. One wonders however to what  
a increasingly fragile GBM whether  
by altered charge or not would lead  
ing and displacement not so much  
ot processes as of the film-like slit  
ms. One would then be dealing, in  
with the same attempt on the part of  
ral epithelial cell to cover the de-  
BM as in the case of the elongation  
ision of a regenerating epithelial cell  
ulcerated surface. The movement of  
processes is associated with the dis-  
it of the glycocalyceal coat from the  
cular surfaces and the slit dia-  
. It is entirely possible that the strings  
d material encountered in the prep-  
of glomerular strands particularly  
6 hr after the injection of NTS may  
ed from such displaced glycocalyces  
rendered more mucoid by the action  
aes which have permeated through  
M or by reduction in its negative

es in the fragility of the glomerular  
an be brought about not only with  
polyamines but with simpler basic  
uch as histamine and 5-OH-trypt-

amine. This is of interest since it is possible  
that these two vasoactive substances can be  
released from mast cells and/or platelets in  
sufficient concentration so as to bring about  
comparable changes in fragility of capillary  
and venular BM. The increased permeability  
observed with these amines would be due  
therefore not only to disjunction of the en-  
dothelial cells allowing the vascular contents  
to come in contact with the BM but would  
also be due to the altered physical property  
and presumably permeability of the BM it-  
self. The vasoactive cationic polypeptides re-  
leased from the lysosomes of polymorpho-  
nuclears and the basic kinins may act on the  
BM in the same way.

*Summary.* Single straight capillary strands  
measuring 60–100  $\mu$ m were secured by micro-  
manipulators and micropipettes from excised  
peripheral portions of the tufts of isolated  
glomeruli. The physical properties of these  
strands were considered to represent those of  
GBM. Normal glomerular strands could be  
stretched up to 40% beyond their initial  
length with good recoil but broke when they  
were stretched from 60 to 77% beyond their  
initial length. By contrast 100% of the glo-  
merular strands from the kidneys with AMN,  
87% of those from kidneys with NTN and the  
most affected glomeruli from aged rats with  
glomerulosclerosis broke immediately when  
stretched beyond their initial length. Elastic-  
ity could not be determined under these cir-  
cumstances. Normal glomerular strands  
showed marked increases in fragility when  
briefly exposed to proteolytic enzymes, neur-  
aminidase, to polycations and to basic  
amines. It seems that direct enzymatic action  
on GBM or alterations in its chemical com-  
position and molecular configuration as well  
as a reduction in its net negative charge can  
bring about striking changes in its fragility.  
These changes appear to be intimately asso-  
ciated with increased permeability. They ap-  
pear with the onset of proteinuria in AMN  
and NTN and they return to near normal  
when the output of urinary protein returns to  
normal. It is suggested that the approxima-  
tion of foot processes which commonly ac-  
companies proteinuric states is a response on  
the part of the visceral epithelial cell to the  
instability and displacement of the foot pro-  
cesses and in particular of the delicate slit

diaphragms occasioned by an underlying increasingly fragile BM. It is also suggested that the vasoactive amines not only lead to disjunction of endothelial cells but render the capillary or venular BM increasingly fragile and permeable.

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## Body Iron Loss in Animals (40343)

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ative information concerning body iron turnover in mammalian species is limited in that turnover rates may differ by orders of magnitude (1, 2). Man appears to have the most restricted exchange, about 1% per day (3), and this may be important in understanding both the high prevalence of iron deficiency (limited absorption) and the occurrence of parenchymal iron overload (limitation). The present study was undertaken to examine body iron turnover in a variety of animal species, since with the exception of the mouse (1) and rat (3), little is known about iron exchange in these other species. It was hoped that these studies would be of general biologic interest but also serve to guide efforts in establishing a mathematical model of parenchymal iron

turnover. The iron content of the diet (490 mg/kg) and a salt mixture which together had an iron content of 512 mg/kg. Weekly food consumption was estimated and its iron content determined by wet ashing (4) and colorimetric analysis. Hematologic measurements including hemoglobin by the cyanmethemoglobin method and hematocrit by the micro technique were made at the beginning and termination of the study. Blood volumes of animals corresponding in weight to those studied were carried out at the beginning of the experiment and at the end in the rat, guinea pig, and rabbit, employing the Evan's blue dye method (5); the blood volume of other species was taken from previous reports in the literature (6-9).

Radioiron ( $^{55}\text{Fe}$ ) in dosage of 1  $\mu\text{Ci/kg}$  (specific activity about 15  $\mu\text{Ci}/\mu\text{g}$ ) was injected intravenously as the citrate salt (20 moles citrate/mole iron) at the beginning of the study, and blood samples were drawn at intervals until the radioactivity had fallen to <30% of the initial level. The interval of sampling was so adjusted that there would be about six samples before this level was reached. At that time an aliquot of all samples was wet ashed, prepared as described by Eakins and Brown (10) and counted in a liquid scintillation counter.

*Animals and methods.* The species studied included the rat, guinea pig, rabbit, dog, monkey, and cow. The first three were from the University of Washington, dogs from the Battelle Northwest facility at Richland, and monkeys were kept in the Primate Research Center at Medical Lake, and the sheep populations were kept at Washington State University. All animals were male. The sheep and cows. Weights of animals were recorded during the experimental period. New Zealand white rabbits were fed Rabbit Breeder Pak which contained 219 mg of iron/kg; Hartley guinea pigs were fed on Guinea Pig Chow by Purina, the iron content of which was 449 mg/kg and given 1 mg of ascorbic acid/100 g/day in their drinking water. Dawley rats were fed Laboratory Chow by Purina with an iron content of 373 mg/kg. Dogs were fed Wayne's Dog Chow with an iron content of 289 mg iron/kg. Fuscicularis monkeys were fed Monkey Chow containing 237 mg iron/kg. Sheep (Columbia ewes) of about 8

Hereford cows of about 12 years old and alfalfa hay which had an iron con-

centration of 490 mg/kg and a salt mixture which together had an iron content of 512 mg/kg. Weekly food consumption was estimated and its iron content determined by wet ashing (4) and colorimetric analysis. Hematologic measurements including hemoglobin by the cyanmethemoglobin method and hematocrit by the micro technique were made at the beginning and termination of the study. Blood volumes of animals corresponding in weight to those studied were carried out at the beginning of the experiment and at the end in the rat, guinea pig, and rabbit, employing the Evan's blue dye method (5); the blood volume of other species was taken from previous reports in the literature (6-9).

In previous studies in man there had been a rapid initial fall, presumably reflecting the mixing of radioiron with nonerythron iron (2, 11). In rats, guinea pigs, and rabbits the eleventh day sample was used as the first point in the turnover curve since that point and those following appear to fall on a single exponential clearance line. In the other species there was a more rapid initial fall, presumably related to mixing with nonerythron body iron. To avoid this mixing phase the start of the turnover slope (called 0 day) was taken after a single exponential rate of decrease in radioactivity was established. In dogs this began at 224 days, in Pigtail and Fuscicularis monkeys 196 days, and in sheep and cows 168 days. Initial and subsequent values for

each animal were plotted, and the best exponential rate of decrease in specific activity was derived by analysis of least squares. The point of intersection with the zero ordinate at the time of the first sample was taken at 100%. Mean  $t_{1/2}$  was established by averaging the individual  $t_{1/2}$  values (Table I) and also by employing the average values at each time interval for each species so as to give a composite turnover curve (Fig. 1). The mean  $t_{1/2}$  was corrected for blood volume changes which occurred as a result of growth, and a further correction was made for blood withdrawn during the experimental period. Total blood removed from rat, guinea pig, rabbit, dog, monkeys, sheep, and cow was 1.2, 2.2, 10, 50, 30, 100, 100 ml, respectively. None of the female sheep or cows became pregnant during the study.

Estimated total body iron (TBI) was calculated from the following formula:

$$\text{TBI (mg)} = (\text{mg Fe/ml whole blood}) \times (\text{ml whole blood}) \times 3/2$$

where the factor 3/2 represents an estimate of the relation of total body iron to red cell iron.

The turnover of body iron (BIT) was calculated according to the formula:

$$\text{BIT (\%/d)} = 0.693 \times 100/t_{1/2}$$

$$\text{BIT (mg/kg/d)} = \text{mg Fe/kg}$$

$$\times \text{turnover (\%/d)}$$

The daily iron intake was calculated from the amount of food consumed and its iron content, as determined by wet ashing and colorimetric analysis. In small animals food intake was monitored over a week; in large animals the food supply over a month's period was estimated. It was assumed that food iron intake/kg remained constant through the study. The % absorption was calculated from the daily turnover of iron plus growth requirements divided by iron uptake.

**Results.** Results of this study are summarized in Table I. The rate of isotope disappearance from circulating red cells in rats, guinea pigs, and rabbits required considerable correction for growth, whereas in the other five species blood volume change was relatively small. Average data points for the corrected rates of isotope turnover in the circulating erythrocytes are shown in Fig. 1. The half-time turnover varied from 138 days in the guinea pig to 761 days in the cow. Corrections were made for weight changes in all species showing increases (dog and cow excluded). Based on estimates of body iron content which varied in different species between 32 and 58 mg/kg, the actual turnover of body iron/kg varied from 220  $\mu\text{g/kg/d}$  in the guinea pig to 41  $\mu\text{g/kg/d}$  in the cow.

The balance sheet for iron requirements in each species, based on change in estimated body iron associated with growth and iron losses is displayed in Table II. These requirements are matched against food iron ingested which varied from about 3 mg/kg/d in the

TABLE I. MEASUREMENTS OF BODY IRON LOSS.<sup>a</sup>

Species	Rat (14)	Guinea pig (7)	Rabbit (7)	Dog (6)	Pigtail (7)	Fusicularis (3)	Sheep (7)	Cow (7)
Sex	M	M	M	M	M	M	F	F
Beginning days <sup>+</sup>	11	11	11	92	9	9	21	21
Hb (g/dl)	16 $\pm$ 0.8**	15 $\pm$ 0.3	13 $\pm$ 0.7	17 $\pm$ 0.5	13 $\pm$ 1.8	12 $\pm$ 1.1	11 $\pm$ 1.7	14 $\pm$ 1.5
Wt (kg)	0.40 $\pm$ 0.02	0.57 $\pm$ 0.01	3.2 $\pm$ 0.08	13 $\pm$ 0.8	9.6 $\pm$ 1.0	6.4 $\pm$ 1.1	80 $\pm$ 12.4	476 $\pm$ 65
BV (ml/kg)	60 $\pm$ 1	60 $\pm$ 4	52 $\pm$ 8	66	61	61	58	57
Ending days <sup>+</sup>	234	234	459	826	812	812	966	966
Hb (g/dl)	16 $\pm$ 0.7	15 $\pm$ 0.5	14 $\pm$ 1.1	17 $\pm$ 1.0	13 $\pm$ 1.6	13 $\pm$ 0.4	14 $\pm$ 1.7	16 $\pm$ 1.8
Wt (kg)	0.67 $\pm$ 0.08	1.2 $\pm$ 0.15	4.5 $\pm$ 0.3	13 $\pm$ 1	11 $\pm$ 2	7 $\pm$ 0.7	85 $\pm$ 11.9	460 $\pm$ 64
BV (ml/kg)	51 $\pm$ 3	54 $\pm$ 3	40 $\pm$ 3	66	61	61	58	57
<sup>55</sup> Fe loss (t½)								
days (uncorr.)	129 $\pm$ 15	91 $\pm$ 8	273 $\pm$ 32	552 $\pm$ 92	383 $\pm$ 146	483 $\pm$ 131	663 $\pm$ 127	761 $\pm$ 205
days (corr.)***	182 $\pm$ 25	138 $\pm$ 15	288 $\pm$ 33		404 $\pm$ 174	452 $\pm$ 246	681 $\pm$ 171	
Estimated miscible body iron (mg/kg)	45	44	32	58	42	42	42	46
Body Iron Turnover								
(%/d)	0.38	0.50	0.24	0.13	0.17	0.15	0.10	0.09
( $\mu\text{g/kg/d}$ )	171	220	77	75	71	63	42	41

<sup>a</sup> Number of animals studied; \*\*SD; \*\*\* corrected for blood volume and hemoglobin change (the underlined value has been used for calculating-iron turnover rate); + days after injection of radioiron.

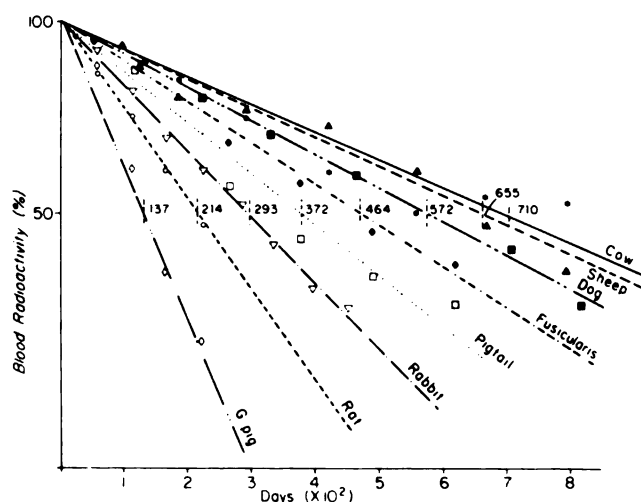


FIG. 1. Red cell radioactivity as a function of time. Symbols used in these studies are as follows:  $\diamond$ —...— Guinea pig;  $\circ$ —...— rat;  $\nabla$ —...— rabbit;  $\square$ —...— pigtail;  $\blacklozenge$ —...— fuscicularis;  $\blacksquare$ —...— dog;  $\blacktriangle$ —...— sheep;  $\bullet$ —...— cow. Each point represents the average activity of the individual groups of animals summarized in Table I. Lines are derived by least squares. The  $t_{1/2}$  in days is indicated for each species.

TABLE II. CALCULATIONS OF IRON ABSORPTION.

Species	Rats	Guinea pig	Rabbit	Dog	Pigtail	Fuscularis	Sheep	Cow
requirements (mg/d)								
growth	0.06	0.19	0.14	0	0.11	0.06	0.31	0
resting	0.08	0.19	0.29	0.94	0.72	0.43	3.5	19
total	0.14	0.38	0.43	0.94	0.83	0.49	3.8	19
food intake (g/d)	15	100	170	345	150	150	3200	8200
iron intake (mg/d)	5.6	44	37	99	36	36	1600	4200
fractional iron Absorption (%)	2.5	0.9	1.2	0.9	0.2	1.4	0.2	0.4

Medium weights used in calculation.

key to as much as 50 mg/kg/d in the Guinea pig. Iron intake by all species was far in excess of iron requirements, so that estimated absorption of food iron ranged between 0.2 to 2.5%.

**Discussion.** In these studies body iron turnover was estimated from the specific activity of radioiron in circulating red cells. Previous studies involving the injection of radioiron intravenously into small animals (3, 12) have shown an initial excessive loss of radioiron through the gastrointestinal tract. In man a very rapid initial fall in red cell activity is observed over the first 300 days due to mixing with body iron stores (2, 11). In order to avoid both of these, turnover was estimated by fitting a single exponential decrease in red cell activity was observed. At this time a steady state of iron exchange within the animal and between the animal and the external

environment was presumed to exist. It was also necessary when following red cell activity to make some assumption concerning the miscible pool in which the isotope was diluted. Studies of iron distribution (12) and stores (3, 13) in the rat and of the miscible pool in man (2, 11) suggest the nonerythron portion to be about one-third of the total. Thus, values for red cell iron were increased by 50% to reflect total body turnover. In addition, adjustments were made for changes in blood volume and total body mass and also for the amount of blood removed for isotope measurements.

In the smaller animals, turnover rates ranged from 0.24 to 0.61%/day. In dogs and monkeys fractional turnover was from 0.15 to 0.19%/day. Sheep and cows showed rates of 0.10 and 0.08%/day. These differences appear to have an inverse relationship to

body weight. Man, however, falls outside of this relationship since daily excretion is 0.03%/day (2, 11). The major difference between man and small animals appears to be in the much greater capacity for excretion of iron through the intestinal mucosal cells in the latter (3, 12, 13). Possibly a variation in this degree of intestinal excretion explains the difference observed in other species.

The data on iron losses also permit estimates of iron absorption. While it might seem more direct to measure absorption itself, this is not practical. Balance studies (food iron ingested minus fecal loss) are not meaningful since the amount of iron ingested is within 1 or 2% fecal iron and contains most of the iron excreted as well as that not absorbed. Isotope studies of absorption must assume similar absorption of isotope and of food iron and are affected by a number of factors which make results highly variable (14). An alternate means of calculating absorption is from the sum of iron requirements for growth and iron losses. The highly favorable ratio between dietary iron and absorption required to maintain iron balance is evident. The estimated absorption range from 0.2 to 2.3% may be contrasted with iron balance in the human. While iron intake in this country is about 150 to 200  $\mu\text{g}/\text{kg}/\text{d}$ , absorption in man is about 12  $\mu\text{g}/\text{kg}$  and in menstruating women about 24  $\mu\text{g}/\text{kg}$  (15, 16). This represents an absorption in the male of about 7% and in the female of about 14% of dietary iron. Obviously, requirements will vary depending on the amount of growth during the period of study. There is also some adjustment of loss in relation to the amount of iron in the diet (1). Thus, both absorption and excretion may be modified somewhat depending on the amount of iron provided. However, the much greater iron intake of all animal species is evident.

**Summary.** Measurements have been made of the decrease in specific activity of radioiron in circulating red cells of eight animal species. From these data calculations of body iron

turnover have been made and establish a general inverse relationship between body size and rate of external iron exchange. A comparison of iron requirements and iron intake in these animals indicate extremely low absorption requirements, ranging from 0.2 to 2.3% of their dietary iron intake.

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## Electrocardiographical, Biochemical and Morphological Effects of Chronic Low Level Cadmium Feeding on the Rat Heart (40344)

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Cadmium is known to dissociate myocardial excitation-contraction coupling and to depress the excitability of the cardiac conduction system *in vitro* (1-7). Short-term feeding experiments concluding that cadmium depresses conduction time through the atrioventricular node-His-Purkinje system have generally extended these *in vitro* observations to *vivo* systems (8, 9). Although the cadmium content of the heart increases with age and exposure (10-14), the effects of cadmium have not been systematically analyzed for possible significance as a contributing factor to degenerative heart disease. This preliminary study was undertaken to investigate electrocardiographical, biochemical, and morphological changes in mammalian hearts related with long-term, low-level cadmium feeding.

**Methods.** Eighteen weanling female rats of Long-Evans strain were obtained and housed as described (15). They received a standard rye-based, low-cadmium diet and aerated water fortified with Mn, Co, Cu, and Mo, as described by Schroeder and Olson (16), for a total of 24 months when all were sacrificed. For half of them, cadmium was added to the water to provide a concentration of five parts per million (ppm) cadmium; for the half which served as controls, no cadmium was added; however, they were otherwise treated identically (16). Of 16 rats (8 cadmium-fed and 8 control), rectal systolic pressures were determined at 8, 18, and 21 months after weaning. Eight rats (4 cadmium-fed and 4 control) were studied electrocardiographically and blood pressures determined directly from which they were sacrificed so that the various parts of the heart could be assayed for cadmium and zinc. The phosphate spectra of hearts from the eight remaining rats

(4 cadmium-fed and 4 control) were analyzed by phosphorus nuclear magnetic resonance spectroscopy (<sup>31</sup>P NMR). In addition hearts from a pair of rats not otherwise studied (1 cadmium-fed and 1 control) were examined by electron microscopy.

**I. Indirect systolic pressure determinations.** At 12, 18, and 21 months after weaning systolic pressure was measured indirectly, as described (17), in rats minimally anesthetized with intraperitoneal sodium pentobarbital, 25 mg/kg of body wt. A tail cuff was slowly inflated to a pressure above systolic and then allowed to deflate slowly until the pulse distal to the cuff reappeared; both the distal pulse and the pressure in the tail cuff were simultaneously recorded on the same graph. A rat's systolic pressure was taken as the median of three measurements made within a period of about a minute.

**II. Electrocardiology.** Eight rats, randomly selected for this phase of the study, were anesthetized with intraperitoneally administered sodium pentobarbital, 35 mg/kg. Lead II of the electrocardiogram (ECG) was monitored during surgery from needle electrodes with a Grass model 7 polygraph. Right femoral artery blood pressures were continuously recorded using a Statham P23 pressure transducer connected to the Grass model 7 polygraph. Systolic pressures recorded with this catheterization technique are reported as the 24-month pressures. The left carotid artery was isolated and catheterized with an insulated silver electrode which served as the active electrode for the His bundle electrogram (HBE) recordings. A reference electrode was placed in the right jugular vein to minimize background noise in the HBE. The active electrode was then positioned to achieve optimal His wave amplitude. The HBE was monitored with an Electronics for



Medicine variable filter amplifier and oscilloscope, model PR6, using standard low and high filter settings, 40 and 500 cycles per sec (cps), respectively (18). Simultaneously, the ECG signal was filtered between 0.1 and 200 cps on a separate channel of the monitor. The electrical events of the heart were recorded with a Tandberg-Honeywell Series 100 FM tape recorder for later replay at one-half speed when they were photographed for analysis on light sensitive paper.

III. *Assay of cadmium and zinc by atomic absorption spectrophotometry.* Following surgery, the hearts from these eight rats were removed, washed in saline, and weighed. The left and right ventricles, upper and lower septum, and atrium were taken. These samples were rinsed in saline, weighed, frozen, and stored in acid-washed plastic containers for later cadmium and zinc assay by atomic absorption spectrophotometry (AAS). Samples were also taken from the left kidney and the liver; these too were weighed, frozen, and stored for metal assay. For the actual assay, aliquots of the heart weighing 0.013–0.770 g, and of kidney and liver, weighing 0.036–0.249 g, were “wet-ashed” by standard techniques, as described (15). The cardiac aliquots were adjusted to final volumes of 2 ml and were then assayed by AAS, using the graphite furnace (19). The renal and hepatic aliquots were adjusted to final vol of 5 ml and were assayed by AAS, using the burner method (15, 19).

IV.  *$^{31}\text{P}$  Phosphorus nuclear magnetic resonance spectroscopy ( $^{31}\text{P}$  NMR).* The eight remaining rats with indirect systolic pressure measurements were heparinized prior to cervical dislocation. The heart from each was then rapidly excised in the cold (0–4°) and immediately immersed in cold Hartmann's modified Ringer solution (4) at pH 7.2. Two 10 ml bolus infusions of this solution, injected through the aorta, washed the remaining blood from the coronary vasculature. After careful dissection to remove connective and adipose tissue from the heart, a sample from the apical region of the left ventricle, was taken for cadmium and zinc assay as described above; samples of the left kidney and the liver were also taken for similar assay. The remaining heart tissue from these rats was weighed and minced while in the cold for  $^{31}\text{P}$  NMR analysis.

Since perchloric acid (PCA) extraction increases the resolution of small tissue samples without altering the phosphate spectra (20, 21), this minced tissue was treated with 1/10 w/v of 60% PCA and homogenized in a Virtis “S” 45 homogenizer for 5 min. The resulting suspension was centrifuged at 10,000 rpm (12100g) for 10 min in a Sorvall superspeed centrifuge, model RC2-B. The pellet was saved for Biuret protein determination, and the supernatant was neutralized to a pH between 7.0 and 7.5, using 10 N KOH in the presence of EDTA.

The neutralized PCA extract was centrifuged at 10,000 rpm (12100g) for 5 min after a 30-min refrigeration period. The supernatant was saved, while the pellet was washed once with 2 ml and thrice with 1 ml of water. The resulting supernatants were combined with the original one and evaporated on a rotary evaporator. Samples were lyophilized overnight and then resuspended in 2 ml of 20% D<sub>2</sub>O.

Spectronic analysis of these PCA extracts was undertaken using a Bruker HFX-5 nuclear magnetic resonance spectrometer with  $^2\text{D}$ -stabilization operating at 36.43 MHz for  $^{31}\text{P}$  (21 kG magnetic field,  $^1\text{H}$  frequency 90.000 MHz). This instrument is equipped with facilities needed for all modes of Fourier transform signal averaging and broad band and continuous wave heteronuclear  $^1\text{H}$  decoupling. Details of the actual conditions and analytical parameters of  $^{31}\text{P}$  NMR spectroscopy have been described (20). Samples were scanned for 2.5 hr.

V. *Electron microscopy.* Representative tissue cubes, 1 mm on a side, of sinoatrial and atrioventricular nodes, left ventricle, right atrium, and septum from nembutal anesthetized, 35 mg/kg, animals were fixed by perfusion with 3% glutaraldehyde in Sorensen's phosphate buffer at pH 7.2. This treatment was followed by osmication in Millonig's fluid, dehydration and embedment in Epon-Araldite. After establishing the morphological orientation with semi-thin sections (1.5  $\mu\text{m}$ ) stained with methylene blue, ultra-thin sections were prepared for electron microscopic viewing. Electron photomicrographs of the several cardiac regions from control and cadmium-fed rats were compared in search of significant anatomical differences.

VI. *Data analysis.* For all ECG and HBE

ngs, a mean was calculated from ten ls measured with a Bausch and Lomb micrometer scaled at 0.1 mm graduation. The paper speed of 150 mm/sec per-resolution to 1 msec. Heart rates were ted from A-A wave interval measure-of the HBE.

dard analytical techniques for the cation of compounds and determina-their concentrations were used (20, 21) yze the  $^{31}\text{P}$  NMR spectra.

ly, concentrations of each phosphate und studied were computed from the the compound peak area to the phosph-primary standard (1.5 mM inorganic hosphate, Na form) peak area. Stu-*t* test was applied to the statistical and variances of each phosphate com-analyzed in the control and cadmium to determine the significance of the ed differences in their concentrations. e of  $P < 0.05$  was accepted as signifi-

electrocardiographical, tissue cad-and zinc concentrations and blood e data were similarly analyzed for sta-significance. Student's *t* test was ap-to the means and variances of control dmium data and a value of  $P < 0.05$  epted as significant.

lis. I. *Systolic blood pressures.* The av-of the indirectly measured systolic es for the eight rats fed 5 ppm cad-exceeded the averages of the control s at 12, 18, and 21 months (Table I). fferences at 18 and 21 months were ally significant ( $P < 0.05$ ); at 12 and 18 months they were not significant but the rs of rats were smaller than we usually ie control average at 12 months was a Hg, while our usual average for such s, approximates 100 mm Hg. The dif-s at 18 and 21 months were in the 15 mm Hg range which 5 ppm cadmium induces under our standard condi-

24-month pressures measured directly rial catheterization averaged 129 mm the four cadmium-treated rats versus n Hg for the four control rats (Table owth rates and total body weights, provide a sensitive measure of toxicity gher doses of cadmium, were the same dmium-fed and control animals

TABLE I. SYSTOLIC BLOOD PRESSURES (mm Hg).<sup>a</sup>

Time (mos)	N	Control	N	5 ppm Cd fed
12	8	108 ± 3	8	117 ± 5 (ns)
18	8	99 ± 4	8	117 ± 7*
21	8	102 ± 5	8	119 ± 5*
24	4	116 ± 6	4	129 ± 8 (ns)

<sup>a</sup> The first three lines cite indirectly measured systolic pressures (mean ± SEM) for eight control and eight cadmium (5 ppm since weaning) rats at 12, 18, and 21 months. The last line cites directly measured systolic pressures (mean ± SEM) for the animals in which electrocardiographic measurements were made at 24 months.

\* Significantly different from control,  $P < 0.05$ .

throughout the entire 24 months of follow-up.

II. *Electrocardiology.* A significant lengthening of the PR interval, despite a more rapid mean heart rate was evident in the ECGs of the cadmium-fed animals (Table II). This depressed excitability was accompanied by a pronounced increase (30%) in the A-H interval of the HBE. Ventricular depolarization time (QS interval) was prolonged (33%) as well, in the treated animals (Table II). Representative ECG and HBE recordings are shown in Fig. 1. These observations suggest that cadmium may potentially depress the excitability of atrioventricular nodal cells (A-H interval prolongation) and may also interfere with ventricular cell to cell conduction (QS interval increase).

Control ECG intervals in this study were comparable to those reported elsewhere (9, 22). Since the HBE intervals are the first reported from rats to our knowledge, valid comparisons with literature values are not possible.

III. *Tissue cadmium and zinc.* The hearts from control rats had cadmium concentrations that approached the minimum measurable levels; while those from cadmium-fed rats had easily measurable levels (Table III). The average cardiac concentrations of the cadmium-fed group ranged from a minimum of 50 (atrial) to a maximum of 900 (lower septal) times the concentrations found in the comparable control samples. Although the absolute cardiac concentrations were small in comparison with those present in renal and hepatic tissues (with the maximum cardiac concentration being about 3% and 10% of the renal and hepatic concentrations, respectively), it is evident that cadmium accumu-

TABLE II. ELECTROCARDIOLOGY.

	N	Rate (complexes/min)	ECG		HBE		
			PR (sec)	QS (sec)	P-A (msec)	A-H (msec)	H-V (msec)
Control	4	234 $\pm$ 23*	0.056 $\pm$ 0.002	0.024 $\pm$ 0.002	8 $\pm$ 1	31 $\pm$ 2	19 $\pm$ 1
5 ppm Cd	4	294 $\pm$ 23	0.073 $\pm$ 0.004**	0.032 $\pm$ 0.001**	9 $\pm$ 1	40 $\pm$ 3	22 $\pm$ 1

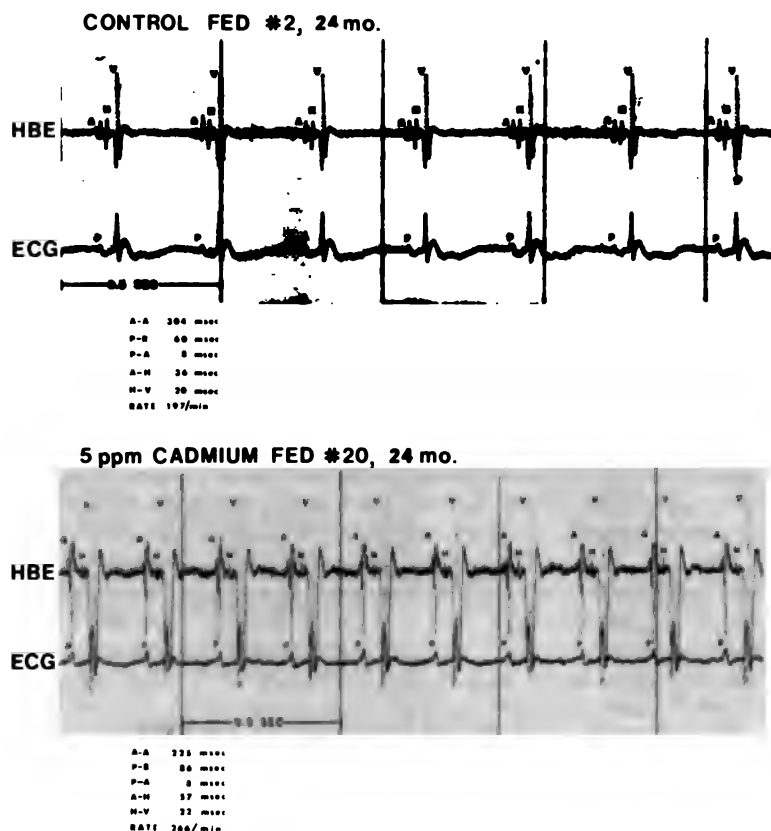
\* Mean  $\pm$  SEM.\*\* Significantly different from control,  $P < 0.05$ .

FIG. 1. Representative of ECG and His Bundle electrogram recordings from control and cadmium-fed rats.

lates in the heart. Moreover these data suggest differences in regional uptake within the myocardium.

Zinc was apparently uniformly distributed within the heart and was not affected by cadmium feeding; thus the heart, unlike kidney or liver (11, 15), did not sequester zinc in response to cadmium feeding. Cardiac cadmium-to-zinc ratios were obviously greatly increased by cadmium-feeding.

IV.  $^{31}\text{P}$  NMR. The integrals of the phosphate spectra for paired heart PCA extracts were analyzed with standard techniques to determine the identity and concentration of

compounds present (20, 21) (Table IV). Significant decreases in ATP and total adenine nucleotide contents were detected in hearts from cadmium-fed animals. Control heart ATP and total adenine nucleotide contents were comparable to those reported elsewhere for rat heart (23, 24). Since the sample for each group ( $N = 4$ ) is small, a detailed interpretation of these observations would be inappropriate at this time.

V. *Electron microscopy.* A preliminary survey of all segments of cardiac tissue removed for microscopic examination revealed that ultrastructural alterations resulting from cad-

TABLE III. TISSUE CADMIUM AND ZINC CONCENTRATIONS.<sup>a</sup>

Tissue	Control			5 ppm Cd Fed		
	Cd ( $\mu\text{g/g}$ wet wt)	Zn ( $\mu\text{g/g}$ wet wt)	Cd/Zn ratio	Cd ( $\mu\text{g/g}$ wet wt)	Zn ( $\mu\text{g/g}$ wet wt)	Cd/Zn ratio
(animals studied by radiography)						
Septum	0.007 $\pm$ .007	10.5 $\pm$ 0.6	0.0007	0.363 $\pm$ 0.21*	9.3 $\pm$ 0.4	0.039
Septum	0.004 $\pm$ .004	13.7 $\pm$ 3.0	0.0003	0.220 $\pm$ 0.09*	10.9 $\pm$ 0.7	0.020
Septum	0.002 $\pm$ .002	11.2 $\pm$ 0.8	0.0002	1.742 $\pm$ 1.61*	9.4 $\pm$ 0.6	0.185
Liver	0.010 $\pm$ .006	12.2 $\pm$ 0.6	0.0008	1.233 $\pm$ 0.95*	10.3 $\pm$ 0.1	0.120
Liver	0.008 $\pm$ .004	11.2 $\pm$ 0.3	0.0007	1.563 $\pm$ 1.154*	14.3 $\pm$ 4.6	0.109
(animals studied by MR spectroscopy)						
Lt. ventricle	0.011 $\pm$ .004	10.4 $\pm$ 1.6	0.0011	0.094 $\pm$ 0.026*	9.8 $\pm$ 1.0	0.010
	0.046 $\pm$ .008	25.3 $\pm$ 1.4	0.0018	45.8 $\pm$ 0.65**	32.2 $\pm$ 1.0**	1.422
	0.011 $\pm$ .003	31.5 $\pm$ 2.4	0.0004	16.8 $\pm$ 2.3**	36.5 $\pm$ 2.1	0.460

in concentrations  $\pm$  standard error of the mean for four control and four cadmium exposed animals in the cardiac samples and eight of each in the case of kidney and liver samples.

\* significantly different from control,  $P < 0.05$ .

\*\* significantly different from control,  $P < 0.01$ .

#### E IV. SELECTED COMPOUNDS DETERMINED BY <sup>31</sup>P NMR.

	( $\mu\text{moles/g}$ Heart wet wt)	
	Control	5 ppm Cd-fed*
Adenine dinucleotide (NAD)	1.3 $\pm$ 0.6	1.4 $\pm$ 0.1
Adenine phosphate phosphocreatine	16.6 $\pm$ 2.2	15.4 $\pm$ 0.9
	4.4 $\pm$ 0.8	2.0 $\pm$ 0.7**
	2.4 $\pm$ 0.5	1.4 $\pm$ 0.4
	2.0 $\pm$ 0.4	1.2 $\pm$ 0.8
Adenosine compounds	8.8 $\pm$ 0.1	4.6 $\pm$ 1.0**
Heart phosphate	40.0 $\pm$ 2.0	32.4 $\pm$ 4.0
(mg/g heart)	126.8 $\pm$ 1.2	127.2 $\pm$ 0.3

Means of two groups of two pooled hearts  $\pm$  SEM. \* significantly different from control,  $P < 0.05$ .

feeding were limited to foci of degeneration within cells of the atrio-ventricular region and apparent increases in densities of intercalated disc membranes in septal tissue (Fig. 2). Sections of sino-atrial atrial and ventricular tissue showed no apparent abnormalities relative to control animals. These findings indicate tentatively absence of ultrastructural changes within atrio-ventricular nodal and septal tissue exposed to low levels (5 ppm) of cadmium in

discussions. Previous *in vivo* and *in vitro* experiments (1, 4, 9) have described the excitatory action of cadmium on the excita-

bility of the myocardial conduction system. This preliminary study provides additional support for these cadmium-induced changes while extending them to include other electrocardiographic changes and changes in cardiac metabolism and morphology. Long-term (24 months) exposure to low-level dietary cadmium (5 ppm in all drinking water) was found to depress electrical events of the heart characterized by a lengthening of mean PR and QS intervals of the ECG, despite a more rapid but comparable mean heart rate. His bundle electrogram analysis suggests that the prolonged PR interval resulted primarily from an increased A-H interval (30%), indicating impaired conduction through the atrio-ventricular node, rather than His-Purkinje cell depression. Although still tentative, the concept of selective distribution of trace elements in cardiac tissue (25) would provide a partial explanation for the apparent specificity of cadmium depression for the atrio-ventricular node of the cardiac conduction system. This hypothesis is further supported by electron microscope evidence showing apparent degenerative cell changes in the atrio-ventricular node.

The extended QS interval (33%), representative of increased ventricular depolarization time, is consistent with the hypothesis that cadmium also may alter ventricular cell excitability and/or obstruct cell to cell conduction. Septal tissue electron micrographs showed apparent marked increases in mem-

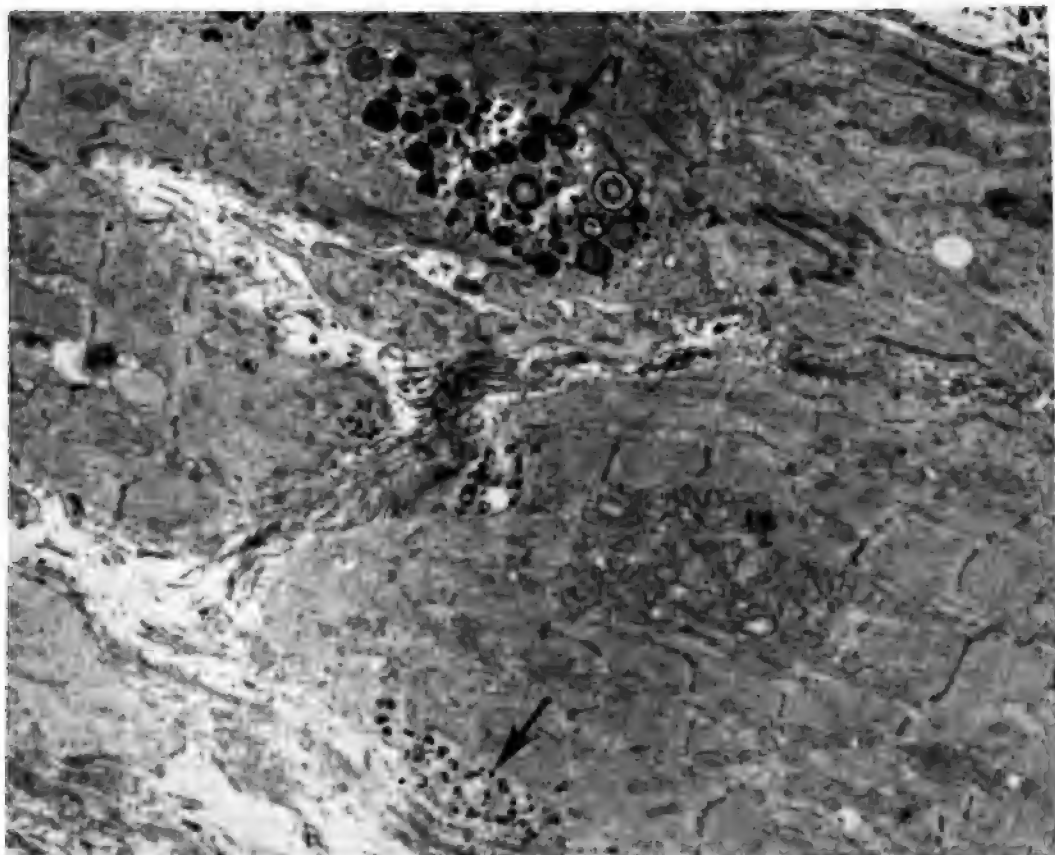


FIG. 2. Cadmium-fed rat, a-v node. Degeneration (arrows) within nodal cells, characterized by an accumulation of dense bodies adjacent to foci of vacuolization. Uranyl acetate and lead citrate. Original magnification  $\times 3200$ .

brane density in the intercalated discs. Since the rapid conductivity of ventricular depolarization is attributed to the intercalated discs, the possibility exists that cadmium may be present in this region bound to membrane structural and/or enzymatic proteins, thereby altering cell to cell conductivity and possibly creating the increases in intercalated disc electron density and mean QS interval of the ECG.

Although the application of  $^{31}\text{P}$  NMR spectroscopy to biomedical research has been a recent development, it has provided an analytical method for rapid characterization of the entire phosphate profile of a given tissue. Such analyses of biopsy samples by other investigators have enabled the detection of subtle metabolic disorders (20, 21).  $^{31}\text{P}$  NMR spectroscopic analysis of heart tissue from animals treated with dietary cadmium revealed metabolic changes consisting most no-

tably of decreases amounting to 57%, 41% and 43% in ATP, ADP and AMP concentrations, respectively. Speculation regarding the mechanism(s) of the altered high energy phosphate metabolism shown to be associated with cadmium treatment in this study, is premature at this time; however, the functional significance may be related to a reduced excitability associated with a decrease in high energy nucleotide content of myocardial tissue, as reported by Opie (24).

In summary, long term exposure to small concentrations of cadmium is associated with depressed myocardial excitability, decreased high energy phosphate content, and morphological changes. The apparent interconsistency between the electrocardiological, biochemical and morphological findings in this study adds credence to the concept that cadmium exposure, either directly or indirectly, compromises the functional integrity of the

heart. Currently, a more detailed study is in progress which will investigate electrical, mechanical, metabolic and morphological changes in mammalian hearts associated with long term, low level cadmium feeding.

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# Central Effect of Somatostatin on the Secretion of Growth Hormone in the Anesthetized Rat<sup>1</sup> (40345)

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Somatostatin was isolated and characterized by Brazeau *et al.* (1) as a hypothalamic tetradecapeptide that inhibits the secretion of growth hormone (GH) from the anterior pituitary. Subsequent studies have revealed that somatostatin is widely distributed in the central nervous system (CNS) (2) and localized subcellularly in nerve ending, synaptosome, in the rat (3). Recently it was also demonstrated in the cerebrospinal fluid in man (4).

Somatostatin has been reported to prolong pentobarbital anesthesia time (5), decrease spontaneous motor activity (6), lower the LD50 of barbiturates and increase strychnine LD50 (7). These results are in contrast to those obtained with thyrotropin releasing hormone (TRH) (5-7). It is possible, therefore, that somatostatin might have a role in the CNS opposite to that of TRH.

We have previously reported that TRH has a dual effects on GH secretion in the anesthetized rat; one is stimulating effect acting directly on anterior pituitary, and another is inhibitory action through the CNS (8). The present study was performed to examine the central effect of somatostatin on GH secretion in the rat.

**Materials and methods.** Male Wistar rats (Japan Animal Co., Osaka) weighing 200-250 g were used throughout the experiment. The animals were maintained in a light (14 hr light and 10 hr dark) and temperature ( $25 \pm 1^\circ$ ) controlled room and fed Oriental Laboratory Chow (Oriental Yeast Co., Tokyo) and water *ad lib*.

After overnight fasting, they were anesthetized with urethane (150 mg/100 g body wt ip) in the morning on the experimental day. Synthetic somatostatin (supplied by Dr. N.

Yanaihara) was dissolved in physiological saline containing 0.24% Fast Green FCF (Chroma Co., Stuttgart) as a dye marker and injected into a lateral ventricle or a pituitary portal vessel of the rat.

In the first experiment, somatostatin (0.5  $\mu$ g and 5  $\mu$ g/rat) was injected into the right lateral ventricle in a volume of 10  $\mu$ l per rat as described previously (9). The same volume of saline solution alone was injected in control animals.

In the second experiment, somatostatin (5  $\mu$ g/rat) of vehicle solution was injected into the lateral ventricle in rats with or without extensive hypothalamic destruction, which was performed two weeks before the experiment with a special knife (stirrup shaped, vertical 2.0 mm, diameter 3.0 mm) as described previously (10) using a modification of the method of Arimura *et al.* (11). The basal medial hypothalamus including the arcuate nuclei and the ventromedial nuclei were necrotically destroyed by interrupting the vascular supply from the ventral surface of the brain.

In the third experiment, somatostatin was injected into a single portal vessel using a modification of the method described by Porter *et al.* (12). Median eminence and pituitary stalk was exposed by the parapharyngeal approach, and fine curved glass cannula was inserted into one of main portal vessels using a micromanipulator. Through the cannula, somatostatin was perfused for 20 min at a flow rate of 25 ng/2  $\mu$ l/min.

In each experiment, immediately before the injection of test materials and at 10-40 min intervals thereafter blood samples of 0.6 ml were withdrawn from the jugular vein using a heparinized syringe as described previously (12).

Plasma GH levels were determined by a specific radioimmunoassay (14) with the ma-

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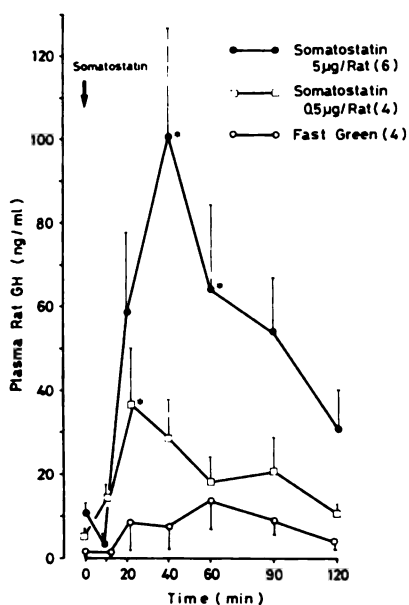


FIG. 1. Effect of intraventricular injection of somatostatin (0.5 and 5  $\mu$ g/rat) on rat plasma GH. Means  $\pm$  SE are shown. Control group was injected with saline containing Fast Green solution alone. The number of animals in each test group is indicated in parentheses. Statistical difference (vs control) is shown by asterisk: \*  $P < 0.05$ .

rial supplied from the National Institute of Arthritis, Metabolism and Digestive Diseases. IAMD-rat GH-RP-1 was used as the reference preparation. Student's *t* test was used for the statistical evaluation.

**Results.** As shown in Fig. 1, injection of somatostatin (0.5 and 5  $\mu$ g/rat) into the lateral ventricle caused a significant and dose-related increase in plasma GH with a peak response at 20–40 min. Initial decrease of plasma GH at 10 min was observed by the injection of a large dose of somatostatin (5  $\mu$ g) but not by a smaller dose (0.5  $\mu$ g).

As shown in Fig. 2, the response of plasma GH induced by intraventricular injection of somatostatin (5  $\mu$ g/rat) was partially, but significantly blunted by extensive hypothalamic ablation compared with those obtained in sham-operated animals (peak GH value: 50.1  $\pm$  8.1 ng/ml vs 100.4  $\pm$  23.6 ng/ml,  $P < 0.05$ ).

Infusion of somatostatin into a stalk–portal vessel for 20 min (25 ng/min) resulted in a significant decrease of plasma GH during the infusion period and no significant change of plasma GH was observed thereafter until 120 min (Fig. 3).

**Discussion.** In the present study, we observed that intraventricular injection of somatostatin resulted in a significant and dose-related increase of plasma GH in urethane-anesthetized rats. GH release induced by intraventricular injection of somatostatin is not restricted to rats anesthetized with urethane, since stimulating effect of somatostatin in-

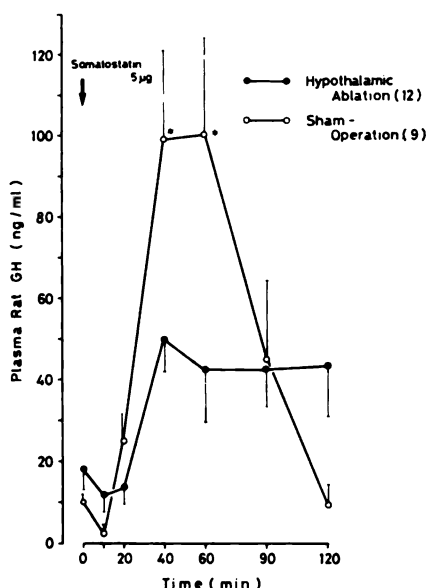


FIG. 2. Effect of hypothalamic ablation on GH release induced by intraventricular injection of somatostatin (5  $\mu$ g/rat). Means  $\pm$  SE are shown. The number of animals in each group is indicated in parentheses. Statistical difference (vs sham-operated group) is shown by asterisk: \*  $P < 0.05$ .

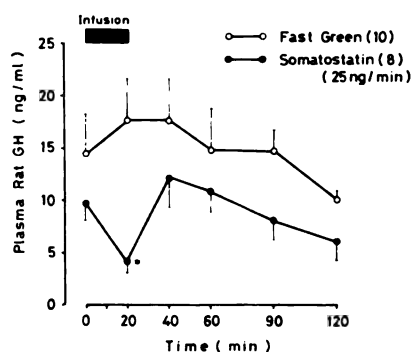


FIG. 3. Plasma GH levels following the infusion of somatostatin (25 ng/min) into a stalk portal vessel for 20 min. Fast Green solution was infused in a control group. Means  $\pm$  SE are shown. The number of animals in each group is shown in parentheses. Statistical difference (vs control) is shown by asterisk: \*  $P < 0.01$ .



jected centrally was also observed in rats anesthetized with pentobarbital or chloral hydrate (unpublished observation). In contrast, injection of somatostatin into a stalk-portal vessel failed to induce GH release. Initial decrease of plasma GH was observed following the administration of somatostatin either intraventricularly or into the portal vessel. The rise in plasma GH following the intraventricular injection of somatostatin cannot be accounted for by a rebound phenomenon following the initial suppression, because the infusion of the peptide into the pituitary portal vessels caused only a slight rebound phenomenon. It appears, therefore, that somatostatin inhibits GH secretion at the pituitary but rather stimulates GH release through the CNS.

These observations are in contrast to the results obtained with TRH (8). TRH stimulated GH release at the pituitary in rats, whereas it has an inhibitory action on GH secretion probably in the CNS. Different CNS effects of these peptides were also previously demonstrated in studies on behavior (7).

The exact mechanism by which intraventricular injection of somatostatin stimulates GH release remains to be investigated. The fact that hypothalamic ablation blunted GH release induced by intraventricular injection of somatostatin suggest that the hypothalamus may play a role, at least in part, in the central effect of somatostatin.

The ventromedial nucleus, which was destroyed by the ablative procedure used in the present experiment, is known to be closely related to GH releasing activity (15). Delayed and long duration of GH response to intraventricular injection of somatostatin is quite compatible to that of various behavioral response which was induced by somatostatin injected into the CNS (16). Cohn *et al.* (17) reported that intraventricular injection of somatostatin induced deep sedation or unusual rotation, which was blocked by atropine. Rezek *et al.* (16, 18) showed that administration of somatostatin into rat amygdaloid or hippocampal formation caused the various behavioral and electrophysiological change. Somatostatin applied by microiontophoresis caused a depressant effect on some central neurons and influenced calcium transport of

cortical synaptosomes (19). These results suggest that somatostatin like other hypothalamic peptides, have a variety of effects on the CNS probably as a neurotransmitter.

It is concluded, therefore, that somatostatin may act somewhere in the CNS as a neurotransmitter to elicit GH release possibly by enhancing GH releasing activity in the hypothalamus, although physiological significance of this central effect of somatostatin must await further clarification.

**Summary.** Injection of somatostatin into the lateral ventricle caused a significant dose-dependent increase in plasma GH in urethane-anesthetized rats. Increases in plasma GH induced by intraventricular injection of somatostatin were significantly blunted in rats with hypothalamic deafferentation. Somatostatin infusion into the pituitary portal vessel significantly lowered plasma GH. These results suggest that somatostatin has dual effects on GH secretion: one inhibitory effect on the pituitary and another stimulating action possibly through somewhere in the CNS.

We are indebted to the National Institute of Arteriosclerosis, Metabolism and Digestive Diseases, Rat Pituitary Assay Program, for supplying the rat GH radioassay kit. We would like to thank Dr. Noboru Chihara, Shizuoka Pharmaceutical College, Shizuoka, for the gift of synthetic somatostatin.

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## Transmembrane Potentials in Bovine Lymphatic Smooth Muscle (40346)

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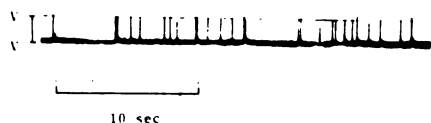
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The use of intracellular electrodes for smooth muscle was introduced by Bülbring and Hooton (1), and this method of recording has since been applied to a variety of smooth muscles. Specifically, intracellular studies of the electrical activity of vascular smooth muscle in the frog have been reported by Funaki (2), in turtle arteries and veins by Roddie (3), and in rat and guinea pig small mesenteric arteries and veins by Trail (4), Speden (5), Nakajima and Horn (6), and Ito and Kuriyama (7). Recently, by means of the sucrose gap method, the present authors have successfully recorded membrane action potentials of bovine mesenteric lymphatics simultaneously with phasic contraction waves which had one-to-one correspondence to the action potentials, and the authors suggested that calcium current may probably play a major role in producing spike discharge in bovine lymphatic smooth muscles (8). The lymphatics exhibited, even *in vitro*, vigorous spontaneous contractile activity. Contractions of the lymphatic smooth muscles were also induced by 5-hydroxytryptamine (5-HT), prostaglandin F<sub>2α</sub>, noradrenaline, histamine, dopamine and acetylcholine. The smooth muscles were particularly sensitive to 5-HT (9). In the following experiments we have studied the membrane activity of single cells of bovine mesenteric lymphatics with intracellular microelectrodes.

**Materials and methods.** Segments of mesenteric lymphatics, between 0.5 and 3 mm in outer diameter, were dissected from the fresh mesenterics of recently slaughtered cattle. Longitudinal strips, about 5 mm long and 1 mm wide, were cut from these segments and kept in a chamber containing a modified Locke's solution of the following composition in mmoles/liter: NaCl 154.0, KCl 5.6, CaCl<sub>2</sub> 2.2, NaHCO<sub>3</sub> 1.8, glucose 5.5. The solution was maintained at 37° and continuously bubbled with 100% O<sub>2</sub>. It was revealed by repetitive direct measurements with a pH meter

(F3, HORIBA) that the solution was kept at an approximately constant pH of 7.4 for more than 6 hr. The strip was mounted with the outer surface upward on a thin silicon rubber plate consisting of the bottom of the chamber. Connective and adipose tissues covering the outermost longitudinal smooth muscle layer were gently removed. A microelectrode filled with 3 M potassium chloride, with tip resistances of about 50–80 MΩ and diameter less than 0.5 μm, was inserted into the wall of the specimen with a micromanipulator under the binocular microscope with incident illumination. A nonpolarizing Ag–AgCl wire was used as a reference electrode. These two electrodes were connected to a high input resistance preamplifier (Nihon Kodens MEZ-8101), the output from which was displayed on a dual beam synchroscope (Iwatsu DS-5015) and recorded by a data recorder (TEAC R-351F).

**Results and discussion.** Spontaneous contractile activity was observed with most of the lymphatic strips under the binocular microscope when incubated in the warm modified Locke's solution. The rhythm of the contractions was regular and highly sensitive to environmental temperature. The beating rate was 4–6/min at 37°. It was almost doubled by the elevation of temperature up to 40°, keeping the specimen length unchanged. Figure 1 shows typical patterns of spontaneous electrical activity in lymphatic smooth muscle. A burst of spike discharges was frequently observed in association with a contraction wave and lasted for several seconds or longer. In this record the resting potential measured at maximum polarization between one spike and another was about –50 mV. The average value of the resting membrane potential was  $-49 \pm 2.4$  mV in 10 experiments. The resting potential sometimes showed slight rhythmic fluctuations or slow waves at various intervals, rarely with an after-hyperpolarization which resembled that in visceral smooth mus-



1. Spontaneous electrical activity in lymphatic muscle.

2). The resting potentials seemed lower in lymphatic smooth muscle with spontaneous activity than in that without the activity. The firing of lymphatic action potentials could be classified into two patterns, i.e. short trains consisting of several spikes, 2) single spikes or irregular spike discharges. The amplitude of the action potentials ranged from 39 to 57 mV (mean  $47 \pm 5$  mV). Occasionally the action potentials showed a slight overshoot of less than 5–7 mV. In some cases, as shown in Fig. 2, many action potentials were superimposed upon the rising phase of the slow fluctuations. The amplitude of the slow waves was about 10 mV and was considerably smaller than in visceral smooth muscle cells (10). The duration of the fluctuations was about 200 msec. Frequently, the discharge appeared to be triggered by the slow depolarization. Figure 3 represents a typical action potential of lymphatic smooth muscles. The configuration of the action potential is similar to that of smooth muscles in the *taenia coli* (1) or portal vein (7) of the guinea pig. The action potential usually consisted of two phases, i.e. rapid depolarization, fast, followed by slow repolarization. The duration of the action potentials was about 40–50 msec at room temperature down to 35°. The prolongation was usually attributed to the extension of the repolarization phase of the action potentials. In the previous paper (8), it was noted that the rhythmicity and amplitude of spontaneous contractions in bovine mesenteric lymphatics were not affected by tetrodotoxin, which is known to be a selective blocking agent for sodium rapid carrier channels. In the present experiments, it was also recognized that the level of resting potentials and the configuration of action potentials were not affected by tetrodotoxin (8a).

The average of resting membrane potentials in lymphatic smooth muscles were lower than those in visceral smooth muscles (12)

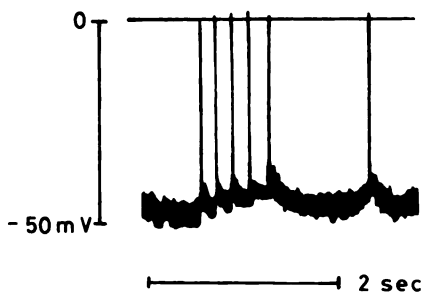


FIG. 2. Lymphatic action potentials superimposed upon the rising phase of slow fluctuations in the resting potential.

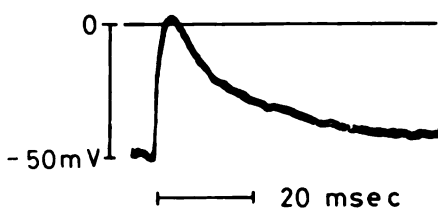


FIG. 3. A typical action potential of lymphatic smooth muscle.

but somewhat higher than those in vascular smooth muscles (4, 5). The lymphatic smooth muscle cell is so small that it may easily be damaged during impalement with a microelectrode. It should be noted, however, that when successful impalement was maintained over a long period of time no change was observed in the value of the resting potential. In the *taenia coli*, Bülbirg (13) noticed that the extension of the smooth muscle, within certain limits, led to an increased tension accompanied by an increased oxygen consumption, and the membrane potential was found to depend upon the degree of stretch. It might be possible that the level of transmembrane potentials could also be influenced by the degree of stretch in lymphatic preparations. In the previous paper (8) it was reported that by means of the sucrose gap technique the average value of resting potentials in lymphatic smooth muscles was estimated to be  $32.7 \pm 4.2$  mV. In the sucrose gap technique, in principle, there are the following controversial points in regard to the estimation of transmembrane potentials: (1) short-circuiting between the electrodes exists in appreciable quantities and (2) the sucrose solution fails to replace all the ions lying in the interstitial spaces. Artifacts due to junction potentials should not be overlooked in

sucrose gap experiments. These may offer an explanation for the differences between the values of resting potentials in lymphatic smooth muscles recorded by the intracellular microelectrode and those by the sucrose gap technique. As represented in Fig. 2, the lymphatic action potentials were frequently superimposed upon the slow fluctuations. When the depolarization due to the fluctuations reaches a critical level, the firing of a single action potential or a burst of spikes may take place. Spontaneous subthreshold fluctuations in membrane potential have been recorded in various muscular and nervous tissues of both vertebrate and invertebrate animals (14, 15). These fluctuations are generally considered to be the basis of rhythmical firing of action potentials and hence of spontaneous mechanical activity. In smooth muscle, subthreshold activity appears to be at least of two kinds. In some cases it is nearly sinusoidal in appearance and is referred to as "slow waves". In other cases the membrane potential depolarizes slowly to a point where threshold is reached and an action potential is initiated. The configurations of the slow fluctuations in lymphatic smooth muscles are similar to those in the rabbit colon (16) or in the guinea pig jejunum (17). It has been reported that spontaneous contractions of visceral smooth muscles are caused by repetitive firing of action potentials. Each burst of spike discharges in lymphatic smooth muscle is well coordinated with the mechanical event amounting to a spontaneous contraction wave under the binocular microscope. The amplitude and duration ( $47.8 \pm 9.4$  msec) of action potentials in lymphatic smooth muscles were lower than those in visceral smooth muscles (12) but somewhat higher than in vascular smooth muscle (4, 5). By use of the sucrose gap technique, the present authors (8) reported that the lymphatic action potentials were similar in appearance to the pacemaker potentials recorded from some other smooth muscles. As a matter of course, the recordings by the sucrose gap technique are extracellular ones and represent compound potentials of a lot of cells present in the preparations. In the present experiments, on the other hand, the transmembrane activities of lymphatic smooth muscles were recorded from the ef-

fector cells located in the margin of the preparations in order to avoid the influence of vigorous spontaneous contractions. The activities were not recorded from the pacemaker sites. This may explain the difference in the configurations of lymphatic action potentials in the present and previous reports.

**Summary.** Transmembrane potentials in smooth muscle fiber of bovine mesenteric lymphatics have been studied with the aid of an intracellular microelectrode technique. Resting potentials ranged from  $-41$  to  $-57$  mV. In most of the preparations, the slow fluctuations in the resting potentials were recognized, amplitude and duration of which were about 10 mV and 400–1200 msec, respectively. A burst of action potentials was associated with a spontaneous contraction wave. The amplitude of the action potentials ranged from 39 to 57 mV. The duration of the action potentials was  $47.8 \pm 9.4$  msec in 10 experiments. The magnitude of occasional overshoot was a few millivolts. The level of the resting potentials and the configuration of the action potentials were not affected by tetrodotoxin.

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## Effect of the Ionophore, A23187, on Contraction and Relaxation of Rat Arteries and Veins (40347)

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Calcium ion plays a crucial role in contraction and relaxation and receptor binding in vascular smooth muscle (1, 2). Many approaches have been used to evaluate the role of calcium in vascular function. The discovery of antiporter ionophores capable of transporting bivalent cations such as calcium across membranes describes a novel approach to improve our understanding of the importance of calcium in the contractile process. The action of the ionophore, A23187, on contraction of certain blood vessels coupled with data on the role of extracellular calcium in this process may disclose important differences in calcium utilization among blood vessels.

Furthermore, use of A23187 may aid in understanding of the mechanism by which norepinephrine relaxes the rat jugular vein (3). We have proposed that beta adrenergic receptor stimulation by norepinephrine may be more pronounced in veins than in arteries (4) with the rat jugular vein providing an extreme example of this phenomenon. Changes in calcium availability in this system must be examined as a potential explanation for the inability of norepinephrine to contract the rat jugular vein. Therefore, in the present study, we compared ionophore-induced changes in responses of the rat jugular vein to those of the femoral vein, a vein that contracts maximally to norepinephrine. The effect of A23187 on the responses of these veins was then compared with the responses in two rat arteries, the aorta and carotid artery.

**Methods. Isolation of vascular tissue.** Male Sprague-Dawley rats (150-300 g) (Harlan Industries, Cumberland, IN) were killed by a blow to the head. External jugular veins, femoral veins, aortas or carotid arteries were dissected free of connective tissue, cannulated *in situ* with polyethylene tubing (PE #50, OD = 0.5 mm) and placed in Petri dishes containing Krebs' bicarbonate buffer (see below).

The tips of two 30 gauge stainless steel hypodermic needles bent into an L-shape were slipped into the polyethylene tubing. Vessels were gently pushed from the cannula onto the needles. The needles were then separated so that the lower one was attached with thread to a stationary glass rod and the upper one was tied with thread to the transducer. This is the procedure for ring preparations (circular smooth muscle) of blood vessels described by Hooker *et al.* (6).

Veins were placed in organ baths containing 10 ml of modified Krebs' solution of the following composition (mM concentrations) except when calcium concentration was varied: NaCl, 118.2; KCl, 4.6;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2.5;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4$ , 1.2; dextrose, 10.0 and  $\text{NaHCO}_3$ , 24.8. This solution was maintained at 37° and aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Initial optimum resting force was 4 g for the arteries and 1 g for the veins (3, 5). Isometric contractions were recorded as changes in grams of force on a Beckman Dynograph with Statham UC-3 transducers and micro-scale accessory attachments. Tissues were allowed to equilibrate 1-2 hr before exposure to drugs.

**Effect of A23187 on contractile responses to norepinephrine, serotonin and potassium chloride.** Cumulative concentration-response curves were obtained from baseline tension by a stepwise increase in concentration after a steady response occurred to the preceding dose. Tissues were then exposed to either A23187 or a solvent control for one hour and then rechallenged with the contractile agonist. Contractile responses were calculated as the change in grams of force for each concentration of agonist. To minimize variability among preparations, maximum response to each agonist before A23187 was considered 100% and contractile responses after A23187 or a solvent control were calculated as a percent of the initial maximum concentration.

in each tissue. In each experiment, the effect of A23187 was compared with a solvent control.

*Effect of A23187 on relaxation responses to norepinephrine, papaverine and nitroglycerin.* Jugular veins were contracted to a moderate degree of tone with serotonin ( $1.78 \times 10^{-7}$  M) or potassium chloride (17–50 mM). Once the contraction reached a plateau, relaxant agonists were added and maximum tissue relaxation for each dose was measured. Relaxation of the contracted tissue back to baseline tension represented 100% relaxation. These studies were then repeated after one hour exposure to A23187 or a solvent control.

*Effect of extracellular calcium on contraction in the rat jugular and femoral veins.* Initial contractile responses were determined as detailed above in 2.5 mM  $\text{CaCl}_2$  in all tissues. Buffer was then changed to Krebs' solution containing 0.825 mM calcium, 0.250 mM calcium and finally no added calcium in the presence of 0.1 mM  $\text{Na}_2\text{EDTA}$ . The calcium concentration of this solution was estimated to be less than  $10^{-6}$  M calcium. In other experiments, buffer was changed to contain 3.75 and 5.0 mM calcium. Contractile responses were repeated after approximately 20 min exposure to each calcium concentration. Maximum contraction at each calcium concentration was expressed as a percent of the response in 2.5 mM calcium.

*Calcium determination.* Total tissue calcium was determined in  $\text{HNO}_3\text{-H}_2\text{O}_2$  digests (7) with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer.

*Drugs used.* All drugs were prepared daily in saline except A23187 and kept on ice during the course of the experiments. A23187 was prepared as an opalescent aqueous solution (8) by dissolving A23187 in 0.5 ml dimethylsulfoxide (DMSO) and diluting with deionized distilled water. The solvent control was prepared in the same way by omitting the A23187. By this technique, the maximum volume of DMSO added to the 10 ml bath was 5  $\mu\text{l}$ . The source of the drugs used was as follows: *l*-arterenol bitartrate (norepinephrine), *l*-isoproterenol-*d*-bitartrate dihydrate, Sterling Chemical Co.; 5-hydroxytryptamine creatinine sulfate complex (serotonin), Sigma Chemical Co.; potassium chloride, Baker Chemical Co.; nitroglycerin U.S.P., papav-

erine hydrochloride, A23187, Eli Lilly and Co.

*Results. Effect in arteries.* The ionophore, A23187 ( $1.5 \times 10^{-6}$  M) did not have a marked effect on the baseline force of either the aorta or carotid artery. In six out of eight aortas, A23187 ( $1.5 \times 10^{-6}$  M) produced a small, slow contraction over 1 hr that was  $14.8 \pm 5.8\%$  of the maximum force generated by the tissue. Three out of seven carotid arteries developed a similar slow contraction over one hour that was  $21.9 \pm 2.0\%$  of the maximum force. The force developed in the presence of the ionophore was dose-dependent. No increase in force was observed in any solvent treated tissues (aorta  $n = 8$ ; carotid artery  $n = 6$ ). Except for this increase in baseline force, responses of aortas and carotid arteries to serotonin, norepinephrine or potassium chloride (Figs. 1 and 2) did not change after A23187 ( $1.5 \times 10^{-6}$  M).

*Effect in veins.* In some jugular veins, A23187 ( $1.5 \times 10^{-6}$  M) produced a slow small contraction over 1 hr but this was not observed in any of the femoral veins examined ( $n = 8$ ). In the femoral vein, A23187 ( $1.5 \times 10^{-6}$  M) exposure for 1 hr decreased the contractile response to norepinephrine and serotonin with a marked reduction in maximum force (Fig. 3). Contraction to potassium chloride, however, was not altered.

Similarly, in the jugular vein, there was a reduction in the maximum force produced by serotonin after A23187 ( $1.5 \times 10^{-6}$  M) (Fig. 4). A23187 did not inhibit contraction to potassium chloride and if anything, produced an enhanced sensitivity to potassium chloride.

Because the rat jugular vein relaxes to many agonists including norepinephrine (3), we examined the effect of the ionophore on vascular relaxation in this issue. After one hour exposure to the ionophore, tissues contracted with low doses of serotonin did not relax completely back to baseline after washing. This was most obvious with  $3 \times 10^{-6}$  M but was observed with concentrations as low as  $0.75 \times 10^{-6}$  M. Even addition of isoproterenol ( $10^{-7}$  M) did not reduce force in such tissues although  $10^{-8}$  M isoproterenol produced a 55% reduction in serotonin-induced force prior to A23187.

After the ionophore, serotonin-contracted

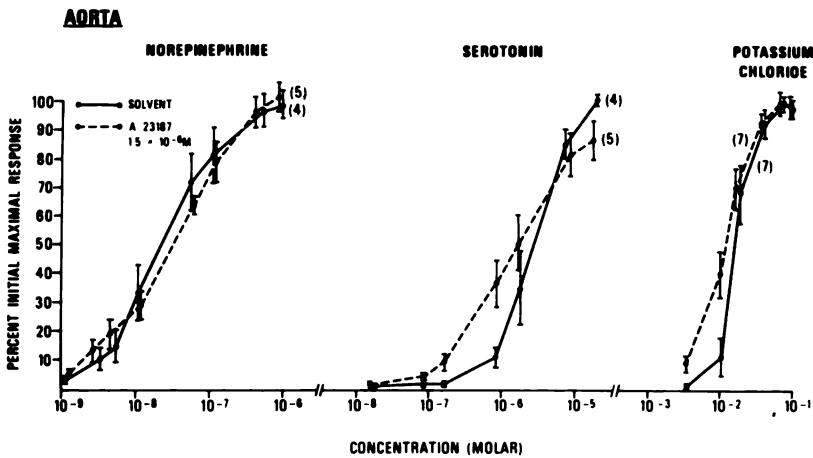


FIG. 1. Effect of A23187 ( $1.5 \times 10^{-6} M$ ) and solvent treatment (see Methods) on rat aortic contraction to serotonin, norepinephrine and potassium chloride. Points are means  $\pm$  SE for the number of tissues in parentheses.

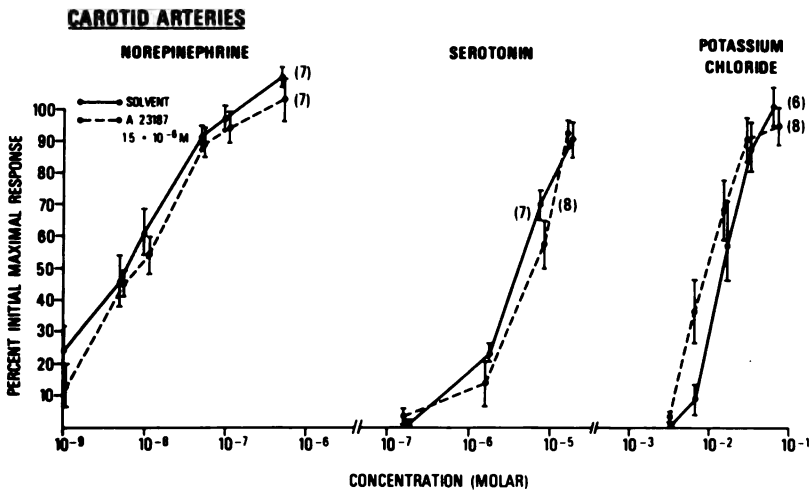


FIG. 2. Effect of A23187 ( $1.5 \times 10^{-6} M$ ) and solvent treatment (see Methods) on contraction of rat carotid arteries to serotonin, norepinephrine and potassium chloride. Points are means  $\pm$  SE for the number of tissues in parentheses.

veins relaxed significantly less to all the relaxant agonists examined; i.e., norepinephrine, papaverine and nitroglycerin (Table I). When jugular veins were contracted with potassium chloride, no difference occurred in relaxation to norepinephrine ( $10^{-5} M$ ) ( $n = 6$ ) or papaverine ( $5 \times 10^{-5} M$ ) ( $n = 7$ ) after A23187 ( $1.5 \times 10^{-6} M$ ). Thus, in the jugular vein, the defective relaxation demonstrated with norepinephrine, papaverine and nitroglycerin may be related to the inhibitory effect of A23187 on serotonin-induced contractions. It is of interest that norepinephrine in concentrations up to  $2 \times 10^{-4} M$  even after A23187 did not contract the rat jugular vein.

*Role of extracellular calcium in venous responses to serotonin and potassium chloride.* Since A23187 differentially affected serotonin and potassium chloride-induced contractions in the jugular and femoral veins, we investigated the role of extracellular calcium in the contraction to these agonists. For comparison, a similar analysis has previously been reported for both serotonin and potassium chloride in the rat aorta (9).

As extracellular calcium concentration was reduced, maximum force developed to both serotonin and potassium chloride declined in jugular and femoral veins (Table II). Decline in maximum response was similar for sero-



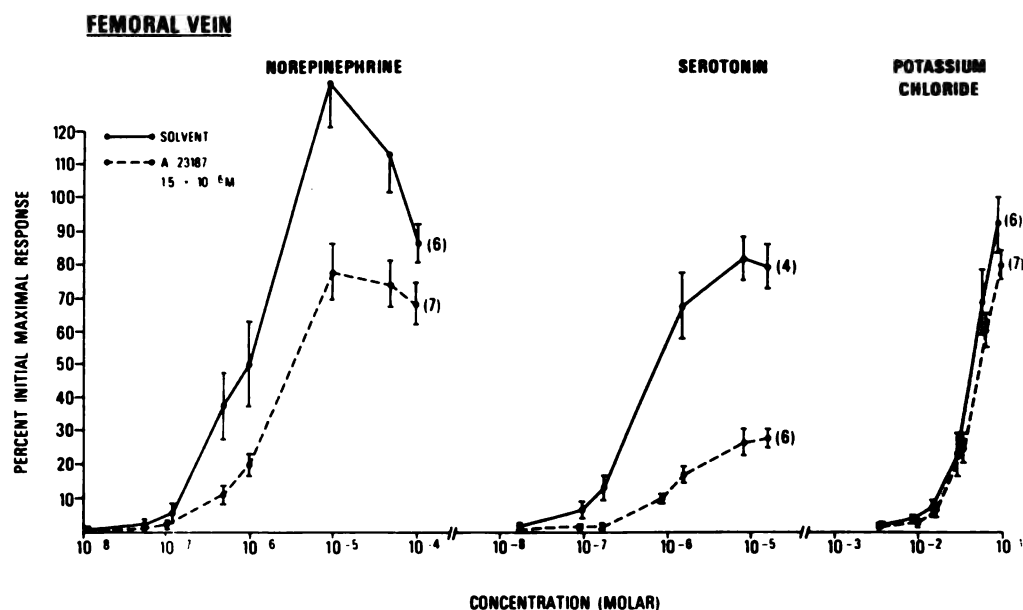


FIG. 3. Effect of A23187 ( $1.5 \times 10^{-6} M$ ) and solvent treatment (see Methods) on contraction of rat femoral veins to serotonin, norepinephrine and potassium chloride. Points are means  $\pm$  SE for the number of tissues in parentheses.

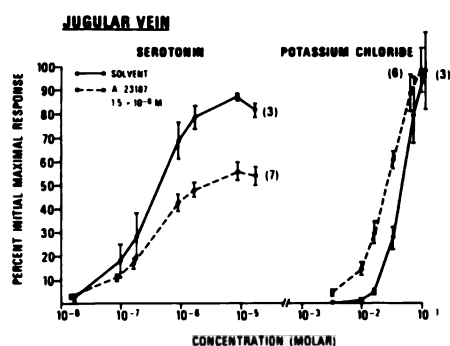


FIG. 4. Effect of A23187 ( $1.5 \times 10^{-6} M$ ) and solvent treatment (see Methods) on contraction of rat jugular veins to serotonin and potassium chloride. Points are means  $\pm$  SE for the number of tissues in parentheses.

tonin and potassium chloride. In the femoral vein, lowering extracellular calcium also reduced responses to norepinephrine, but reduction in norepinephrine contraction was less than for serotonin and potassium chloride at 0.825 mM and 0.250 mM calcium. In jugular veins, higher calcium concentrations did not significantly affect the maximum response to either serotonin or potassium chloride (Table II).

**Tissue calcium.** Total calcium did not differ between jugular veins ( $0.055 \pm 0.004 \mu\text{eq Ca}^{2+}/\text{mg dry tissue}$ ;  $n = 11$ ) and femoral

veins ( $0.058 \pm 0.007 \mu\text{eq Ca}^{2+}/\text{mg dry tissue}$ ;  $n = 7$ ).

**Discussion.** The effect of the calcium ionophore, A23187, on vascular smooth muscle has not been widely studied. The present investigation in both rat arteries and veins confirms the slow and minimal direct contractile effect of A23187 shown on aortic tissue (10). The lack of a marked contraction of rat blood vessels to A23187 is in contrast to its reported contractile effectiveness in guinea pig fundus, taenia coli (11), ileum (12), bronchi (13), atrium (8) and the stomach muscularis from *Bufo marinus* (14). Other smooth muscle preparations such as the vas deferens (12) have been reported not to contract to A23187. Differences in the contractile effectiveness of A23187 in various smooth muscles is consistent with the concept of differences in the calcium availability or utilization among such tissues.

Another way to evaluate an action of A23187 in vascular tissue is to determine its effect on contractile responses to other agonists. No enhancement of maximum contraction to serotonin, norepinephrine or potassium chloride occurred in any vessel examined. The use of A23187 in vascular tissue revealed two major findings: (1) A23187 rather than enhancing contractile responses,

TABLE I. EFFECT OF A23187 ON RELAXANT RESPONSES IN RAT JUGULAR VEIN.

	Control	After solvent <sup>a</sup>	After A23187 ( $1.5 \times 10^{-6}$ M) <sup>a</sup>
		<i>Percent relaxation<sup>b</sup></i>	
Norepinephrine ( $10^{-6}$ M)	51.9 $\pm$ 6.8 (6)	64.5 $\pm$ 7.3 (6)	21.2 $\pm$ 6.4 (6) <sup>c</sup>
Papaverine ( $10^{-5}$ M)	46.1 $\pm$ 6.8 (6)	65.8 $\pm$ 8.8 (5)	-4.0 $\pm$ 7.7 (3) <sup>c</sup>
Nitroglycerin ( $10^{-7}$ M)	44.2 $\pm$ 5.0 (6)	39.6 $\pm$ 3.2 (5)	18.9 $\pm$ 5.2 (3) <sup>c</sup>

<sup>a</sup> Solvent (see Methods) or A23187 were in contact with the tissue for 1 hr.

<sup>b</sup> Tissues were contracted to a moderate tone with serotonin ( $1.8 \times 10^{-7}$  M) and when contraction reached a plateau, relaxant agonist was added. Relaxation was measured three min later. Values are means  $\pm$  SE for the number of tissues in parentheses.

<sup>c</sup> Relaxation was significantly less ( $P < .05$ ) than control relaxation as determined with Student's *t* test.

TABLE II. EFFECT OF EXTRACELLULAR CALCIUM CONCENTRATION ON THE CONTRACTILE RESPONSES OF RAT JUGULAR AND FEMORAL VEINS TO POTASSIUM CHLORIDE, SEROTONIN AND NOREPINEPHRINE.

	Extracellular calcium concentration (mM)					
	0 <sup>a</sup>	0.25	0.825	2.5	3.75	5.0
	<i>Percent contraction in 2.5 mM calcium<sup>b</sup></i>					
<i>Jugular vein</i>						
Potassium chloride (4) (130 mM)	27.6 $\pm$ 6.5	49.3 $\pm$ 2.7	58.8 $\pm$ 3.6	100	93.3 $\pm$ 6.2	99.4 $\pm$ 6.8
Serotonin (4) ( $9 \times 10^{-6}$ M)	0.6 $\pm$ 0.2	23.7 $\pm$ 3.1	54.8 $\pm$ 8.1	100	81.7 $\pm$ 4.8	81.8 $\pm$ 2.4
<i>Femoral vein</i>						
Potassium chloride (7) (130 mM)	15.2 $\pm$ 1.6	19.1 $\pm$ 1.4	45.7 $\pm$ 3.9	100	—	—
Serotonin (7) ( $9 \times 10^{-6}$ M)	1.6 $\pm$ 0.9	33.0 $\pm$ 3.2	49.9 $\pm$ 3.6	100	—	—
Norepinephrine (5) ( $10^{-5}$ M)	5.3 $\pm$ 2.7	51.0 $\pm$ 11.8	72.1 $\pm$ 10.2	100	—	—

<sup>a</sup> Buffer contains no added calcium in the presence of 0.1 mM Na<sub>2</sub> EDTA.

<sup>b</sup> Values are means  $\pm$  SE for the number of tissues in parentheses.

selectively inhibited the maximum force developed to serotonin and norepinephrine but not to potassium chloride, and (2) this effect only occurred in the two rat veins examined and not in the aorta or carotid artery.

We considered the possibility that in veins, contractile responses to serotonin and norepinephrine might utilize tissue calcium stores that differ from those utilized or mobilized during contractile responses to potassium chloride and that dependence on extracellular calcium in veins differed from arteries. However, our data indicate that serotonin and potassium chloride both rely on extracellular sources of calcium in veins, yet only the response to serotonin was reduced after A23187. Additionally, norepinephrine dependence on extracellular calcium in the femoral vein was similar to the aorta (9, 15-17),

yet A23187 did not affect aortic responses. Thus, there was no correlation between dependence on extracellular calcium and the inhibitory effect of A23187 in veins. The possibility that in veins, A23187 produced a large increase in intracellular calcium, that actually inhibited the response to serotonin or norepinephrine is also unlikely. When extracellular calcium was raised in the jugular vein, response to serotonin was not markedly inhibited as occurred with A23187.

Differences in calcium utilization between jugular and femoral veins have been proposed to explain the opposite effects of norepinephrine in these tissues, i.e., norepinephrine relaxed the rat jugular vein (3) and contracted the femoral vein (5). Since both veins responded similarly to A23187 and to manipulation of extracellular calcium, calcium uti-

lization does not appear to differ between the jugular and femoral veins. Furthermore, we considered the possibility that total calcium levels may be lower in the jugular vein than in the femoral vein. However, there was no difference in calcium levels between these veins. Thus, although based on indirect evidence, we propose that differences in calcium handling do not provide a satisfactory explanation for the unusual responsiveness of the rat jugular vein.

**Summary.** The present study describes differences in the effect of the ionophore, A23187, on contraction and relaxation in certain rat arteries and veins. A23187 selectively inhibited maximal contraction to receptor agonists such as serotonin and norepinephrine in veins but not arteries. Furthermore, based on the role of extracellular calcium, the action of A23187 and measurement of total calcium levels, no difference in calcium handling was apparent between the rat jugular and femoral veins. Therefore, relaxation of the rat jugular vein to norepinephrine is probably unrelated to any uniqueness in calcium utilization.

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# The Effect of Glucocorticoid Antagonizing Factor on Hepatoma Cells (40348)

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Endotoxin poisoned animals are refractory to hydrocortisone induced glucose synthesis and glycogen deposition in the liver (1). Endotoxin also inhibits the hydrocortisone induced synthesis of several hepatic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), one of the key enzymes in gluconeogenesis (2). The cortisol antagonist from endotoxin-poisoned animals, glucocorticoid antagonizing factor (GAF), is a heat and dialysis sensitive serum borne factor believed to be released by the poisoned host's macrophages (3).

*In vivo* studies of GAF are difficult, and its precise quantitation has not been possible. To overcome problems inherent in animal studies, an *in vitro* system involving cultured hepatoma cells was developed. Reuber H35 rat hepatoma cells have been adapted for study of the endotoxin-cortisol antagonism. These cells are responsive to corticosteroids and retain fully inducible PEPCK activity (4) even when endotoxin is added directly to the culture. However, when serum from endotoxemic animals or the supernatant fluid from a peritoneal macrophage culture is added to the hepatoma cells sufficient GAF is present to block cortisol induced PEPCK synthesis. Thus, hepatoma cells are suitable for the study of the endotoxin induced cortisol antagonist, GAF. The present report makes the availability of these cells evident.

**Materials and methods. Animals.** Specific pathogen free CD1 mice of both sexes 8-10 weeks old were employed. They were given food and water *ad libitum*.

**Enzyme induction.** PEPCK induction was induced in mice by injecting subcutaneously into the interscapular region one mg of hydrocortisone acetate (cortisol, Sigma Chemical Co., St. Louis, MO) suspended in 0.1 ml of sterile saline containing 0.0025% benzalkonium chloride (Sigma). PEPCK synthesis was

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also induced by two ip injections at 90 min intervals of 500  $\mu$ g N<sup>6</sup>-O<sup>2'</sup>-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (dibutyryl cyclic AMP, Sigma) plus 1 mg theophylline (Sigma) dissolved in sterile saline.

**Endotoxin.** Endotoxin extracted from *Salmonella typhimurium*, SR11, by the method of Westphal and Jann (5) was dissolved in sterile nonpyrogenic saline (Travenol Labs, Deerfield, IL) for injection.

**Cell culture conditions.** A cloned line (KRC7) of Reuber H35 cells derived from the H35 rat hepatoma (6, 7) were obtained from Dr. W. D. Wicks, Department of Pharmacology, University of Colorado Medical Center, Denver. Experimental cultures were grown as monolayers in 60  $\times$  15 mm glass petri plates in Dulbecco's modified Eagle's medium (KC Biological, Inc., Lenexa, KS) containing 5% fetal bovine serum, 5% calf serum, 50  $\mu$ g/ml streptomycin and 75 units/ml penicillin G. Stock cultures were passaged by trypsinization every week. All cultures were grown in a humidified incubator at 37° in an atmosphere of 5% CO<sub>2</sub>-95% air. The medium was changed once on day 4 and cells were used when confluent, i.e., after 7-8 days of subculture (200,000 cell inoculum per plate).

**Experimental conditions for cell culture.** When cells were used experimentally, Dulbecco's medium was replaced with serum-free medium of the same formulation and left overnight (ca. 12 hr). At this time, fresh serumless medium was added along with inducers and inhibitors that were dissolved in the same serum-free medium. The final volume of medium was 5 ml/plate. Stock solutions of hydrocortisone-21-sodium succinate, N<sup>6</sup>-O<sup>2'</sup>-dibutyryl adenosine 3',5'-cyclic monophosphoric acid, and theophylline were added to cultures to give final concentrations of 1  $\mu$ M, 0.5 mM, and 1 mM, respectively.

**Enzyme assays.** Enzyme activity was measured in the cytosol fraction of H35 cells. Hepatoma monolayers were washed with sa-

line and suspended in 1.0 ml of 0.15 M KCl, 0.001 M EDTA, pH 7.6. Cells were fractured by three cycles of freeze-thawing in a dry ice-acetone bath and the cytosol fraction was isolated by centrifugation for 20 min at 20,000g at 4°. PEPCK activity was measured by the  $\text{NaH}^{14}\text{CO}_3$  fixation assay as described by Ballard and Hanson (8). The cytosol activity of tyrosine aminotransferase (TAT) was determined by the method of Diamondstone (9). Protein concentration was determined by the method of Lowry *et al.* (10). Hepatic PEPCK activity was determined by the method of Phillips and Berry (11).

**Collection of serum.** Serum from endotoxin treated mice was collected 2 hr after iv injection of 50  $\mu\text{g}$  endotoxin, then filtered through 0.45  $\mu\text{m}$  filters (Millipore Corp., Bedford, MA) and stored at  $-20^\circ$ .

**Reticuloendothelial system activation.** Mice were primed for serum GAF production by pretreatment with Zymosan-A (Sigma). Priming of mice consisted of 3 iv injections, the first of 0.5 mg and the others of 1.0 mg zymosan given on consecutive days. Serum was collected 48 hr after the last zymosan dose.

**Leucocyte preparations.** Peritoneal exudate cells (PEC) were collected four days after ip injection of 3 ml of NIH thioglycollate broth (Difco Lab, Detroit, MI) by peritoneal washing with 3 ml of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free phosphate buffered saline (PBS), pH 7.4. Cells were centrifuged (500g, 15 min) and resuspended in serumless Dulbecco's medium for culture at  $37^\circ$  in 5%  $\text{CO}_2$ -95% air. Nonadherent cells were removed by washing after 2 hr incubation. Adherent cells were incubated further in fresh media. Cell viability was determined by dye exclusion of the vital dye, trypan blue. Cell numbers were determined by direct count in a hemacytometer (American Optical Corp., Buffalo, NY).

**Collection of conditioned medium from macrophage cultures.** Adherent cells from mouse PEC were cultured in serumless media with or without 10  $\mu\text{g}/\text{ml}$  endotoxin for 24 hr. The supernatant fluid was collected and concentrated 10 $\times$  by ultrafiltration (Millipore Immersible Molecular Separator). Remaining salts and small molecules were removed by elution of the concentrate through Bio-gel P-6 (Bio-Rad Lab., Richmond, CA). Protein

rich fractions were pooled and reconcentrated to the original concentrate volume. Concentrates were filter sterilized through 0.45  $\mu\text{m}$  filters and stored at  $-20^\circ$ .

**Statistics.** Statistical significance between means was determined by the rank-sum test of White (12).

**Results.** The inhibition by endotoxin of cortisol induced PEPCK synthesis is believed to be a mediated effect (1, 3). Direct evidence for this hypothesis is presented in Table I which shows that endotoxin has no inhibitory effect on induced PEPCK synthesis in cultured hepatoma cells exposed to either hydrocortisone or to dibutyl cyclic AMP.

GAF-rich serum from zymosan treated endotoxin-challenged mice (ZES) when injected into endotoxin tolerant mice inhibits PEPCK induction (13). A similar response is seen in hepatoma cells (Table II). Addition of this serum to a final concentration of 2% in the culture medium totally blocks cortisol induced PEPCK synthesis but has no effect on induction of the enzyme by dibutyl cyclic AMP. Similar results were obtained with hepatoma cells when rat serum was the source of GAF. ZES does not significantly inhibit TAT synthesis *in vitro* (Table II) nor does endotoxin inhibit cortisol induced TAT synthesis *in vivo*.

Normal mouse serum possesses some background inhibitory activity and produces a small reduction in cortisol induced enzyme synthesis. This is seen from the data in Table III. Serum from normal mice given endotoxin

TABLE I. INDUCTION OF PEPCK IN ENDOTOXIN TREATED HEPATOMA CELLS.

PEPCK Activity $\pm$ SEM <sup>a</sup>			
Inducer added to medium			
Treatment	None	1 $\mu\text{M}$ Hydrocortisone	0.5 mM Dibutyl cyclic AMP + 1.0 mM theophylline
None	40 $\pm$ 2 (6) <sup>b</sup>	96 $\pm$ 2 (6)	90 $\pm$ 2 (6)
Endotoxin 10 $\mu\text{g}/\text{ml}$	41 $\pm$ 2 (6)	94 $\pm$ 2 (6)	88 $\pm$ 2 (6)

<sup>a</sup> Mean activity as units (nmoles  $\text{NaH}^{14}\text{CO}_3$  fixed/min) per mg protein  $\pm$  SE of the mean for 8-hr induction period.

<sup>b</sup> Number of samples.

## E II. INDUCTION OF PEPCK AND TAT IN HEPATOMA CELLS EXPOSED TO SERUM WITH GAF ACTIVITY.

added to medium	PEPCK activity <sup>a</sup>		TAT activity <sup>b</sup>	
	Control cells	ZES treated <sup>c</sup> cells	Control cells	ZES treated <sup>c</sup> cells
rtisone 1	39 ± 2 (6) <sup>d</sup> 75 ± 3 (6)	41 ± 1 (6) 39 ± 1 (6)	36 ± 2 (6) 276 ± 10 (6)	140 ± 9 (6) 249 ± 9 (6)
1 cyclic 0.5 mM + ylline. 1	71 ± 5 (6)	72 ± 5 (6)	—	—

n activity as units (nmoles NaH<sup>14</sup>CO<sub>3</sub> fixed/min) per mg protein ± SE of the mean for 8-hr induction

n activity as units (μg *p*-hydroxyphenylpyruvate formed/10 min) per mg protein ± SE of the mean for 8-hr 1 period.

osan primed mice challenged with endotoxin, 2 hr serum added to 2% (v/v) in medium.

number of samples.

## E III. INDUCTION OF PEPCK IN HEPATOMA EXPOSED TO NORMAL SERUM AND SERUM FROM ENDOTOXIN POISONED MICE.

ons to medium	PEPCK activity <sup>a</sup>	
	Control	1 μM hydro-cortisone
mouse serum	57 ± 8 (6) <sup>b</sup>	105 ± 8 (6)
1)	—	85 ± 6 (6)
in serum <sup>c</sup> (0.1	—	70 ± 9 (6)

n activity as units (nmoles NaH<sup>14</sup>CO<sub>3</sub> fixed/mg protein ± SE of the mean for 8 hr induction. number of samples.

m collected 2 hr post 50 μg endotoxin iv.

## LE IV. PEPCK INDUCTION BY DIBUTYRYL CYCLIC AMP IN ENDOTOXIN POISONED MICE.

nment	PEPCK activity <sup>a</sup>	
	Cortisol treated <sup>b</sup> mice	Dibutyryl cyclic AMP treated <sup>c</sup> mice
224 ± 7 (7) <sup>e</sup>		206 ± 12 (6)
in <sup>d</sup> 111 ± 12 (7)		207 ± 15 (6)

vity expressed as μmoles PEP/g dry wt liver/6 SE of the mean. Assays performed 4 hr after injection.

g sc.

ses of 500 μg ip dibutyryl cyclic AMP + 1 mg lline.

μg ip 6 hr prior to enzyme induction.

number of mice.

kedly more inhibitory than normal set is less inhibitory than ZES (Tables

III). Base levels of PEPCK are not antly affected by endotoxin or serum

addition over the 8-hr incubation period. Basal TAT activity was elevated after addition of serum from endotoxin poisoned mice. Altered serum insulin levels may account for this effect (4). Neither endotoxin nor serum samples were cytotoxic for the hepatoma cells for the duration of the experiments.

Dibutyryl cyclic AMP induced PEPCK synthesis is unaltered in both hepatoma cells exposed to ZES and in mice poisoned with endotoxin (Table IV).

Figure I demonstrates that GAF-rich serum (ZES) diluted step-wise produces progressively less inhibition of PEPCK in H35 cells. Thus a 50% inhibitory dose (ID<sub>50</sub>) of serum can be determined as the amount that produces 50% inhibition of control PEPCK induction by hydrocortisone. Serum pools were titrated accordingly for GAF activity with the results shown in Table V. Normal mouse serum contains a titer of from 2–8 ID<sub>50</sub>'s. Endotoxin challenge increases the titer to 13, while serum from zymosan primed mice has an average titer of 28.

As little as 0.025 ml (0.5%) of GAF-rich serum consistently produced a significant inhibition of PEPCK induction in hepatoma cells. Injection of at least 10 times this much serum is required to detect enzyme inhibition in mice (13).

Hepatoma cells were used to confirm *in vivo* experiments (3) showing production of GAF by macrophages. Supernatant fluids from adherent mouse PEC were collected 4 and 24 hr after the addition of endotoxin to macrophage cultures. Concentrated fluids

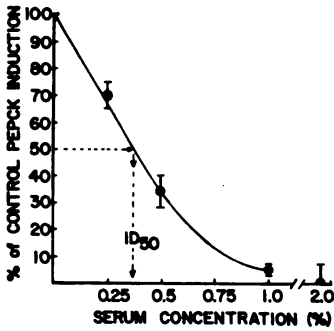


FIG. 1. Titration of serum glucocorticoid antagonizing activity in hepatoma cells. Serum was collected from zymosan pretreated mice 2 hr after challenge with 50  $\mu$ g iv endotoxin. The serum pool was diluted in culture medium in a stepwise manner and added to hepatoma cultures. Values represent PEPCK activity expressed as a percentage of control (no serum addition) induction after 8 hr exposure to 1  $\mu$ M hydrocortisone. Each point represents the mean for four observations  $\pm$  SE of the mean.

TABLE V. TITERS OF GAF IN MOUSE SERUM.

Source of serum <sup>a</sup>	Titer ID <sub>50</sub> /ml <sup>b</sup>
Normal mice:	
untreated	6 $\pm$ 2 (5) <sup>d</sup>
endotoxin treated, 50 $\mu$ g iv, (ES)	13 $\pm$ 1 (6)
Zymosan treated mice <sup>c</sup> :	
untreated	6 $\pm$ 1 (3)
endotoxin treated, 50 $\mu$ g iv, (ZES)	28 $\pm$ 4 (7)

<sup>a</sup> Serum collected at 2 hr after endotoxin.

<sup>b</sup> ID<sub>50</sub> = amount of serum inhibiting control PEPCK induction by 50%.

<sup>c</sup> Zymosan treated mice received 0.5 mg, 1.0 mg, and 1.0 mg of zymosan iv on days 4, 3, and 2 prior to endotoxin challenge.

<sup>d</sup> Number of serum pools titrated.

from both unpoisoned and poisoned macrophages significantly inhibited PEPCK induction in hepatoma cells (Table VI). Fluids from unpoisoned cells may inhibit induction because of GAF release as a result of physical manipulation of the cells or because endotoxin contaminated the glassware. It is significant that the inhibition seen with the macrophage product is specific for PEPCK since TAT remains inducible in hepatoma cells exposed to macrophage supernatant fluids.

**Discussion.** Endotoxin suppresses cortisol induced enzyme synthesis by stimulating a secondary inhibitor, GAF. Cortisol induced PEPCK synthesis in cultured hepatoma cells has now been found to be a valuable assay

TABLE VI. EFFECT OF MACROPHAGE CULTURE SUPERNATANTS ON PEPCK AND TAT INDUCTION IN HEPATOMA CELLS.

Additions to medium	PEPCK Activity $\pm$ SEM <sup>a</sup>	TAT Activity $\pm$ SEM <sup>a</sup>
Controls:		
None	39 $\pm$ 3 (6) <sup>d</sup>	61 $\pm$ 3 (6)
1 $\mu$ M Hydrocortisone	80 $\pm$ 4 (6)	554 $\pm$ 46 (6)
1 $\mu$ M Hydrocortisone + Macrophage supernate from:		
4 hr untreated cells <sup>c</sup>	58 $\pm$ 1 (6)	529 $\pm$ 28 (6)
4 hr endotoxin treated cells <sup>c</sup>	51 $\pm$ 3 (6)	495 $\pm$ 19 (6)
24 hr untreated cells	64 $\pm$ 3 (6)	628 $\pm$ 34 (6)
24 hr endotoxin treated cells	50 $\pm$ 1 (6)	580 $\pm$ 27 (6)

<sup>a</sup> Mean activity as units (nmoles NaH<sup>14</sup>CO<sub>3</sub> fixed/min) per mg  $\pm$  SE of the mean for 8 hr induction.

<sup>b</sup> Mean activity as units ( $\mu$ g *p*-hydroxyphenylpyruvate formed/10 min) per mg protein  $\pm$  SE of the mean for 8 hr induction.

<sup>c</sup> Adherent mouse PEC cultured with or without 10  $\mu$ g/ml endotoxin. 10 $\times$  concentrated and desalted supernate from 1.27  $\times$  10<sup>7</sup> cells added to 10% (v/v) concentration on hepatoma cells.

<sup>d</sup> Number of samples.

for GAF activity. Particular advantages of this *in vitro* assay over *in vivo* assays include insensitivity to endotoxin and detection of 5–10 times less GAF than that detectable by hepatic enzyme responses in mice.

GAF as assayed in hepatoma cells is specific for cortisol induced PEPCK synthesis since it has no effect on dibutyryl cyclic AMP induced PEPCK synthesis. Cortisol is thought to induce PEPCK synthesis by stimulating DNA transcription and production of new messenger RNA, while dibutyryl cyclic AMP is believed to stimulate translational steps of PEPCK synthesis (4). GAF, therefore must block production of new specific messenger RNA but not alter the translation of existing messenger RNA. GAF does not appear to block cellular entry of cortisol since TAT remains fully inducible by cortisol. Interaction of GAF with specific cortisol receptors or receptor sites for hormone-receptor complexes in the nucleus has not been examined. Results with the hepatoma system indicate that GAF lacks species specificity between mice and rats.

Until now, assays for GAF were possible

*in vivo* and no satisfactory dose response could be achieved (13). Hence, precise quantitation of GAF was impossible. The ability to quantitate serum GAF by titration in hepatoma cells provides a valuable tool for analyzing the responsiveness of various animals to endotoxin. It is significant that zymosan-treated mice show higher GAF titers than normal mice following endotoxin challenge. Zymosan and other agents which produce splenomegaly sensitize to endotoxin lethality (14) and to rapid hypoglycemic shock. If GAF reduces gluconeogenesis by inhibiting PEPCK synthesis and possibly that other enzymes in the gluconeogenic pathway, then animals sensitized to the lethal effects of endotoxin should have elevated responses as zymosan-treated mice do. The detection of GAF activity in culture medium from adherent mouse peritoneal cells confirms the proposed lymphoreticular localization of GAF (3, 13).

The presence of GAF-like activity in normal serum may indicate a role for GAF as a general metabolic and immunologic regulator. Adrenal cortical steroids are powerful modulators of immune responses so that their administration could be advantageous under conditions of stress (i.e., infection) when enormous cortisol is released. Cultured hepatoma cells have potential uses for assay of cortisol production in animals following infection or endotoxin poisoning; quantitative assay for the purification of GAF; and qualitative analysis of the mechanism of cortisol action.

**Summary.** Glucocorticoid antagonizing activity of GAF, from cultured macrophages and serum of endotoxemic mice blocks cortisol induction of phosphoenolpyruvate carboxylase in Reuber H35 rat hepatoma cells. Endotoxin treatment of hepatoma cells

was not inhibitory. Dibutyryl cyclic AMP induced enzyme synthesis and cortisol induced synthesis of tyrosine aminotransferase were not affected by GAF. Phosphoenolpyruvate carboxylase induction by cortisol in hepatoma cells could be used to quantitate levels of GAF in serum. This assay system is ten times more sensitive than *in vivo* assays for GAF and it can also be used to titrate serum samples for comparing GAF responses.

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## Effects of Adrenalectomy on Thyroid Function and Insulin Levels in Obese (ob/ob) Mice (40349)

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The finding that hypophysectomy prevents the development of obesity in the genetically transmitted obese (ob/ob) mouse (1) and in the fatty (Zucker) rat (2) has focused attention on possible abnormalities of the hypothalamic-endocrine systems of these animals (3-7). Detailed evaluation of the pituitary-thyroid system has shown no significant abnormalities (8). The reproductive system, however, is immature and pituitary-gonadal feedback is abnormal (9-11). The pituitary-adrenal axis may also be impaired. The adrenal glands are larger (12, 13) and circulating concentrations of corticosterone are higher in ob/ob mice (14, 15). Adrenalectomy reduces the accelerated weight of the ob/ob mouse (16, 17) and improves glucose tolerance (16). Whether these effects are due primarily to adrenalectomy or to the associated reduction in food intake is not known since no pair-gained control mice were used. The present paper reports the effects on body weight, glucose, and insulin concentrations of adrenalectomy in ob/ob mice with that in pair-gained control mice.

**Methods and materials.** *Animals.* The 42 lean and 41 obese (C57B1/6J-ob) mice used in these experiments were purchased from the Jackson Laboratories, Bar Harbor, Maine. The lean animals included both heterozygotes (+/ob) and homozygotes (+/+). They were fed Purina Laboratory Chow (Ralston Purina Company, St. Louis, MI).

**Experimental procedures.** Experiment 1. Fourteen lean and 12 obese animals were bled at 14-15 weeks of age and adrenalectomized 10 days later through a flank incision under ether anesthesia. The experiment was terminated after 34 days. Following adrenalectomy, animals were maintained on 10 µg/day of hydrocortisone sodium succinate and 1% sodium chloride in their drinking water.

Experiment 2. Twenty-eight lean and 29 obese animals were adrenalectomized at 5-6

weeks of age. Hydrocortisone was only used during the early postoperative period. From the third day onward, adrenalectomized animals received 1% sodium chloride as their drinking water but no corticosteroids. Animals were maintained at  $25 \pm 1^\circ$  with a 12-hr cycle of light and dark.

One group with sham-operated obese animals were pair-gained to the adrenalectomized obese animals and another group allowed to eat ad libitum. Pair-gaining was accomplished by giving each mouse 2.2 g/day food and with extra food added or withheld to adjust slightly upwards or downwards for differences in body weight. Blood samples were obtained from the retro-orbital sinus. Animals were fasted for 4 hr prior to sacrifice in exp. 1. In exp. 2 they were bled twice, initially after an overnight fast and 34 days later after a 4-hr fast which followed 1 hr of access to food following an overnight fast. Radioactive  $^{131}\text{I}$  (2 µCi) was given 4½ hr prior to sacrifice. Blood was obtained at autopsy and the thyroid, liver, stomach, and salivary glands were removed, weighed and radioactivity assayed by placing tissues in glass tubes and then into a well type scintillation counter. Insulin was assayed by a double antibody radioimmunoassay technique (18) using rat insulin as a standard and iodinated pork insulin as the competitive binder. Glucose was measured by the glucose oxidase method. Statistical comparisons used the Student's "t" test for grouped data.

**Results.** Experiment 1. The 5 month old obese (ob/ob) mice lost weight following adrenalectomy. At the time of sacrifice, the body weights of adrenalectomized obese animals had declined from  $47.9 \pm 1.8$  g to  $38.1 \pm 1.2$  g. By matching the weight of a group of obese sham-operated controls to that of the adrenalectomized animals the effects of reduced food intake could be taken into account. The lean animals showed an initial dip in body weight after adrenalectomy but sub-

sequently regained it. At autopsy the uptake of  $^{131}\text{I}$  by the thyroid of the lean adrenalectomized animals was not significantly higher than in the lean sham-operated pair-gained group. In the adrenalectomized ob/ob mice  $^{131}\text{I}$  uptake was similar to that in the lean mice. Intact pair-gained ob/ob mice had lower (but not significantly different) uptake of  $^{131}\text{I}$ . Radioactivity in the blood as a percent of the injected dose was significantly higher in the obese adrenalectomized mice than in the obese pair-gained controls (Table I). Prior to surgery the insulin concentrations in the obese (ob/ob) mice were 955 ng/ml compared to 6.5 ng/ml for the lean animal. Adrenalectomy and pair-gaining of ob/ob mice reduced the concentration of insulin to levels that were comparable to those of the lean animals prior to surgery.

**Experiment 2.** The body weight of the sham-operated obese mice which were fed ad lib rose more rapidly than in the sham-operated lean animals (Fig. 1). Adrenalectomy reduced the rate of weight gain in the obese mouse to nearly parallel that of the lean adrenalectomized or sham-operated controls. During the 56 days from adrenalectomy to the time of the first bleeding the lean adrenalectomized animals gained  $4.3 \pm .6$  g (Table II). The adrenalectomized obese mice gained  $6 \pm 1.1$  g which was not significantly greater than the adrenalectomized lean mice. Sham-operated lean animals gained  $5.5 \pm 0.4$  g whereas the sham-operated obese mice gained  $19 \pm 0.5$  g. At the time of the first bleeding, pair-gained obese animals had been fasted overnight. When the radioiodine was given with the initiation of four hours fast

(Table II) the uptake in the neck region in vivo of the adrenalectomized lean animals was significantly higher than in the neck of the sham-operated obese animals. When the experiment was repeated 34 days later the pair-gained animals had been fasted overnight but feeding was allowed for 1 hr prior to the injection of  $^{131}\text{I}$ . When the animals were allowed to eat *ad libitum* for 1 hr the uptake of radioactive iodine was significantly lower in the pair-fed than in the adrenalectomized animals. Animals with low uptake of  $^{131}\text{I}$  had higher urinary iodide excretion than

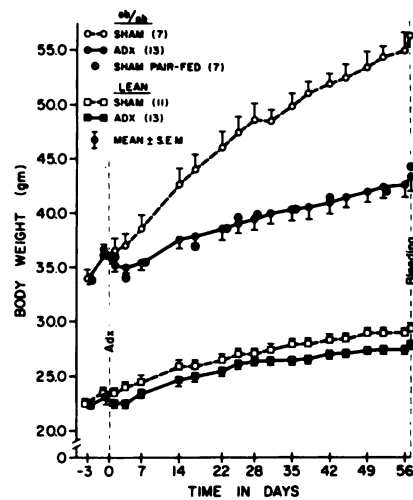


FIG. 1. Weight gain after adrenalectomy. Weight gain over 56 days was measured in sham-operated and adrenalectomized lean or obese mice. A group of sham-operated obese mice were pair-gained to the adrenalectomized animals. The SE of the mean (SEM) is indicated by a line either above or below the point representing the mean.

TABLE I. BODY WEIGHT, THYROID FUNCTION AND INSULIN LEVELS OF OBESE (ob/ob) AND LEAN MICE 6 WEEKS AFTER ADRENALECTOMY OR PAIR-GAINING.

	Lean		ob/ob		<i>P</i> <sup>a</sup>
	Sham	ADX	Sham Pair-gained	ADX	
Change in body wt (g)	1.7 ± 0.3 <sup>b</sup>	0.2 ± 1.2	-7.3 ± 3.9	-9.8 ± 3.3	<.01
<i>P</i>	N.S.		N.S.		
Thyroid uptake $^{131}\text{I}$ (% of injected dose)	18.9 ± 3.5	23.5 ± 4.8	12.7 ± 3.3	25.1 ± 6.1	N.S.
<i>P</i>	N.S.		N.S.		
Blood radioactivity (% of injected dose)	0.41 ± 0.13	0.49 ± 0.17	0.27 ± 0.10	0.75 ± 0.11	N.S.
<i>P</i>	N.S.		<.01		
Insulin (ng/ml)	2.8	1.0	5.7	4.0	

<sup>a</sup> Comparison of adrenalectomized groups which were 18-19 weeks old at the beginning of the study.

<sup>b</sup> Mean SEM.

animals with higher uptake. The sham-operated obese mice had insulin values that were nearly 50 times higher than the insulin levels of the lean sham-operated animal (Table III). Adrenalectomy reduced the level of insulin in the obese mice as did pair-feeding in sham-operated animals. This suggests that most of the hyperinsulinemia of the ob/ob mouse is secondary to the increased food intake and weight gain in the free-feeding animal. Hyperglycemia of the free-feeding sham-operated obese mouse was significantly higher than in the adrenalectomized or sham-operated pair-fed obese mouse and only slightly higher than that of the lean animals.

*Discussion.* Phenotypic expression of obe-

sity and hyperphagia in the obese (ob/ob) mouse is profoundly influenced by the pituitary-adrenal axis. The reduction in weight gain and lowered blood glucose has been reported previously (14, 16) but our observations on thyroid function and insulin have not. The inclusion of a control group of ob/ob mice that were fed only enough food to produce changes in body weight similar to those of the adrenalectomized ob/ob mice allows us to distinguish between effects which are attributable to hyperphagia and those due to adrenalectomy. From the two experiments it appears that the reportedly lower uptake of radioactive iodine by the thyroid of the ob/ob mice (8) may result in part from hyperactivity

TABLE II. WEIGHT GAIN AND THYROID FUNCTION OF LEAN AND OBESE (ob/ob) MICE 9 WEEKS AFTER ADRENALECTOMY OR PAIR-GAINING.

	Lean		ob/ob			P <sup>a</sup>
	Sham	ADX	Sham	ADX	Sham pair-gained	
Change in body wt (g)	5.5 ± 0.4 <sup>b</sup>	4.3 ± 0.6	19.0 ± 0.5	6.0 ± 1.1	8.5 ± 1.5	N.S.
P		N.S.		<.01	N.S.	
Thyroid study I (neck count)	8.4 ± 1.2	25.2 ± 1.4	21.6 ± 2.3	29.6 ± 2.3	30.1 ± 2.5	N.S.
P		<.05		<.05	N.S.	
Blood count (% dose)	0.26 ± 0.02	0.37 ± 0.03	0.31 ± 0.05	0.45 ± 0.05	0.87 ± 0.13	N.S.
P		<.05		N.S.	<.05	
Urine radioactivity (% dose)	65.0	46.0	47.0	30.9	30.7	
Thyroid Study II (% dose)	11.1 ± 0.9	9.9 ± 1.1	7.4 ± 0.7	5.5 ± 0.2	2.9 ± 0.7	<.01
P		N.S.		<.05	<.01	
Blood count (% dose)	0.26 ± 0.01	0.30 ± 0.03	0.31 ± 0.08	0.33 ± 0.05	0.10 ± 0.02	N.S.
P		N.S.		N.S.	<.01	
Urine radioactivity	39.5 ± 3.5	46.4 ± 2.2	46.0 ± 7.4	37.4 ± 3.5	70.5 ± 5.3	N.S.
P		N.S.		N.S.	.01	

<sup>a</sup> Comparison of lean and obese adrenalectomized animals which were 5-6 weeks old at the beginning of the experiment.

<sup>b</sup> Mean ± SEM.

TABLE III. INSULIN AND GLUCOSE OF OBESE (ob/ob) AND LEAN MICE NINE WEEKS AFTER ADRENALECTOMY OR PAIR-GAINING.

	Lean		Obese (ob/ob)			P <sup>a</sup>
	Sham	ADX	Sham	ADX	Sham Pair-Gained	
Insulin (ng/ml)	4.1 ± 0.9 <sup>b</sup>	5.2 ± 1.5	218.0 ± 7.5	81.4 ± 20.3	70.5 ± 11.5	<.01
P		N.S.		<.01	N.S.	
Blood sugar (mg/dl)	50.5 ± 1.5	41.5 ± 0.8	189.5 ± 20.8	88.8 ± 7.9	63.0 ± 11.0	<.01
P		<.01		<.01	N.S.	

<sup>a</sup> Comparison of adrenalectomized lean and obese animals which were 5-6 weeks old at the beginning of the experiment.

<sup>b</sup> Mean ± SEM.

drenal glands with increased losses of n the urine.

changes in glucose and insulin were entirely the result of reduced food Starvation and food restriction are to restore responsiveness to insulin *vivo* (19) and *in vitro* (20). Our findings that the effects of adrenalectomy in g glucose and insulin (17) toward nor- the result of reduced food intake. copolous and Jeanrenaud (4) have ar- at many of the metabolic changes in /ob mouse can be explained by the ned levels of insulin. Thus an expla- of the hyperinsulinemia is central to derstanding of the obese (ob/ob)

e effects of adrenalectomy on hyper- and weight gain in the ob/ob mouse : explained in two ways. Catechol- injected directly into the brain can te food intake (21). This effect is lly reduced after adrenalectomy and is ed over control levels by the injection icosteroids (21). Lowering corticoste- y adrenalectomy might reduce the hy- gic effects of endogenous brain cate- ines which are known to be increased entration in the brain of the ob/ob (22). A second explanation is related oposed enzymatic basis for the genetic in the ob/ob mouse (23). It has re- een suggested that a deficiency of the -inducible component of the sodium rt system in the cell membrane may : an enzymatic basis for the obesity in nimals (23). The ouabain-inhibitable  $(K^+)$ -ATPase is involved in the reup- catecholamines in the brain, the step is involved in termination of action. ncy of this enzyme at this site might hance the action of catecholamines on

nary. The effects of adrenalectomy on se mouse were compared using ani- hich were weight-matched by con- food intake. Adrenalectomy reduces gain of obese (ob/ob) mice. The re-

duced insulin and glucose after adrenalectomy are largely the result of reduced food intake. Changes in thyroid function are related to both the changes in food intake and to adrenalectomy itself.

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# The Long Term Effect of Estrogen Administration on the Metabolism of Male Rat Bone<sup>1</sup> (40350)

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There is considerable information about the effect of estrogen upon bone metabolism in the female including recent work (1) outlining in some detail the long-term effects of the hormone on various parameters of bone metabolism. Because of well known sex differences in the incidence of metabolic bone disease and because it became apparent that there were some differences in the response of bones of male and female animals, the following experiments were carried out in order to determine the long term effect of estrogen on the bones of male rats.

**Materials and methods.** Hundred and fifty-gram male rats were divided into four groups. The animals of the first group were left intact and served as the control group. Group 2 included intact animals treated with 400 µg per 100 g body wt of 17-β-estradiol in sesame oil twice a week. The hormone was introduced directly into the gastric lumen. The rats in group 3 were surgically castrated and those in group 4 were castrated and treated with the same dosage schedule of 17-β-estradiol. The animals in the control groups received similar amounts of sesame oil without hormone. Five rats in each group were sacrificed by decapitation for each set of chemical determinations at 1, 3, 6, 9 and 12 months following the institution of therapy. Serum was collected for chemical determinations. The femora and tibiae were removed immediately and dissected free of soft tissues and periosteum. The epiphyses were discarded and the bone marrow was removed by flushing with a cold saline solution. The metaphysis was separated from the diaphyseal portion of the bone in a standard fashion and only metaphyseal bone was used for chemical analysis. Body weights were recorded monthly. Serum calcium and phosphorous determinations were carried out in an auto-analyser.

The following determinations were carried out on bone: The pooled metaphyses of a single animal were lyophilized and used for each set of determinations. The lipids were extracted and washed according to the method of Folch *et al.* (2). The ash content was determined after ashing a sample of dried defatted bone powder in a furnace at 680° for 20 hr. The hydroxyproline content was measured in an aliquot of fluid from a sample which had been hydrolyzed in 6 N HCl at 100° for 17 hr according to the method of Stegemann (3). Hexosamine was estimated after hydrolysis in 3 N HCl at 100° for 17 hr by a modification of the method of Boas (4) with omission of the resin treatment. Incubation studies were carried out according to the method of Deiss *et al.* (5). Minced metaphyseal fragments were incubated in buffered Krebs-Ringer bicarbonate medium at pH 7.4 in a Dubnoff incubator under 95% oxygen, 5% CO<sub>2</sub> at 37° for 4 hr. The incubation medium contained either 10 µCi of L-proline <sup>14</sup>C with a specific activity of 232 mc/-mole or 10 µCi of D-glucose-[<sup>14</sup>C] with a specific activity of 4.06 mc/-mole. After incubation, the bones were washed with saline and cold water several times and hydrolyzed at 100° for 17 hr with 6 N HCl for hydroxyproline or with 3 N HCl for hexosamine. The <sup>14</sup>C hydroxyproline was isolated by paper chromatography and the specific activity of the hydroxyproline fraction determined according to methods previously described. In order to determine the specific activity of <sup>14</sup>C hexosamine the hydrolysate was applied to an ion exchange resin (Dowex 50W) according to Boas (4). An aliquot was dissolved in 15 ml of aquasol (New England Nuclear, Boston, MA) and the radioactivity determined in a liquid scintillation counter. The degree of quenching was estimated by internal standardization and the data corrected.

Collagenolytic activity was determined according to the method of Kaufmann (3). 50 mg of metaphyseal bone was cut into four

<sup>1</sup> Supported by Grant No. MA 1571, Medical Research Council of Canada.

d placed in a tube containing 100  $\mu$ l of neutral soluble rat skin collagen with  $^3\text{H}$  proline and  $^3\text{H}$  hydroxyproline (approximately 5,000 cpm) with 400  $\mu$ l of 0.1 M Tris-HCl buffer at pH 7.5. They were incubated at 35° for 3 days and the hydrolytic activity of the bone was determined by counting the release of radio-activity into the medium. Blank values were obtained by parallel incubation of metaphyseal bone at 100° for 3 min.

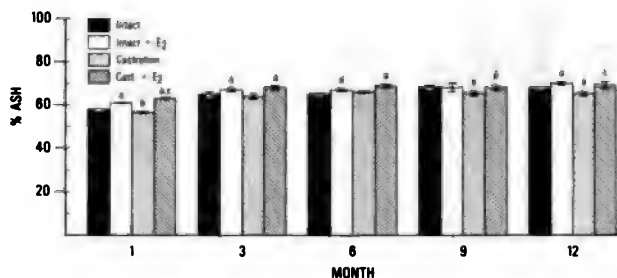
To judge the uptake of mineral by bone, rats were injected intravenously with 1  $\mu$ Ci of calcium-45 containing 2  $\mu$ M EDTA. Five days after injection, the rats were sacrificed by decapitation and the tibiae were removed immediately. The tibia was removed as before and the distal end of the tibia was separated and placed in a furnace at 680° for 20 min. The ashed metaphysis was dissolved with 10 ml of aquasol and counted in a scintillation counter.

**Results.** Estrogen administration to the intact rat caused a consistent and sustained decrease in body weight. Castration also caused a decrease in body weight and estrogen administration appeared to have no significant effect upon this parameter, although when administered to the castrated animal, there was a suggestion of further decrease in weight.

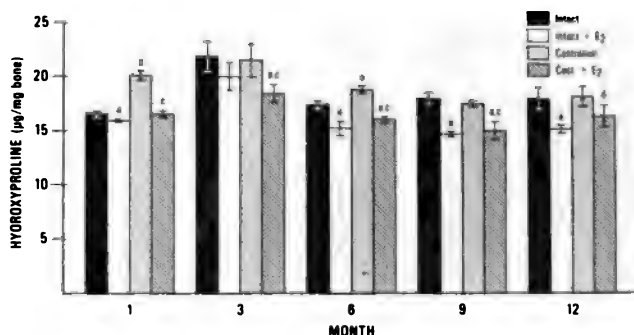
There was no influence of either castration or estrogen administration on serum calcium or phosphorous.

Estrogen administration to the intact animal caused a small but significant increase in the bone ash content and this was sustained over the entire 12 month period (Fig. 1). Castration caused a small but significant decrease in the ash content which was still present at 12 months and estrogen administration returned this value to normal.

Estrogen administration to the intact animal caused a significant and sustained decrease in the total hydroxyproline content of bone (Fig. 2). Castration caused an initial



**Percent ash of dry bone.** The bars represent the mean value and the SE are illustrated. Significant difference between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Difference between the castrated animals and the estrogen treated castrated animals are indicated by (c)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.



**Hydroxyproline content of bone.** The bars represent the mean value and the SE are illustrated. Significant difference between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Difference between the castrated animals and the estrogen treated castrated animals are indicated by (c)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.

increase in bone hydroxyproline content at 1 month but by 12 months there was no difference between the castrated and intact animals. Estrogen administration to the castrated animal did cause a sustained decrease in bone hydroxyproline. Hydroxyproline incorporation rates (Fig. 3) indicated that estrogen administration to the intact animal caused a decrease in the uptake of radioactive proline in bone. Castration caused no significant change and estrogen administration to the castrated animal also decreased the synthesis rates of bone collagen. Castration appeared to decrease the total bone hexosamine (Fig. 4) value at 6 months but at 12 months, the value had returned to normal. Estrogen administration to the intact animal appeared at 12 months to have increased the bone hexosamine content. The specific activity of bone hexosamine (Fig. 5) was decreased when estrogen was administered to the intact animal.

Castration had no effect but estrogen administration to the castrated animal also caused a decrease in the value.

Estrogen administration to the intact animal caused a decrease in the uptake of radioactive calcium into bone (Fig. 6). Castration also appeared to cause a decrease and estrogen administration to the castrated animal caused a further decrease in this value.

There was no significant effect of estrogen administration on bone collagenolytic activity of male rat bone.

**Discussion.** There appear to be several significant differences when one compares this data with that derived from a similar study of the female rat (1). In the first place, removal of the ovaries in the female leads to a decrease in serum calcium and estrogen replacement returns this to normal. Secondly, data in the female indicated that removal of the ovaries causes an increase in bone turn-

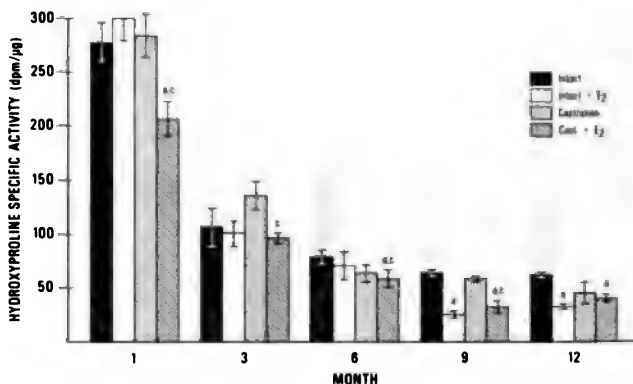


FIG. 3. *Hydroxyproline specific activity of bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c)  $P < 0.01$ , or (d)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.

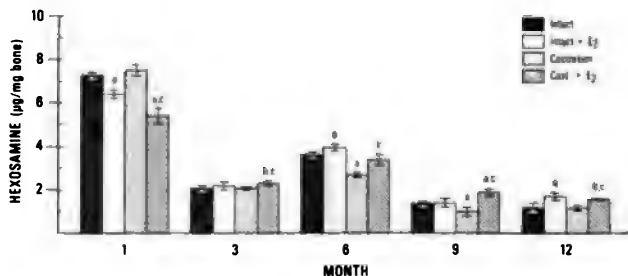
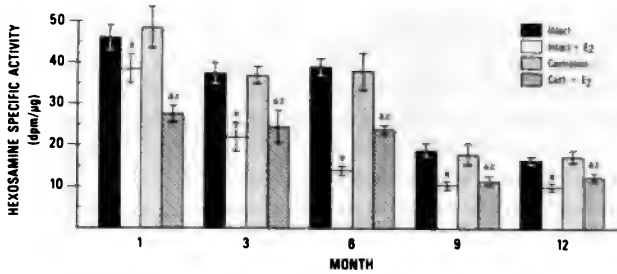
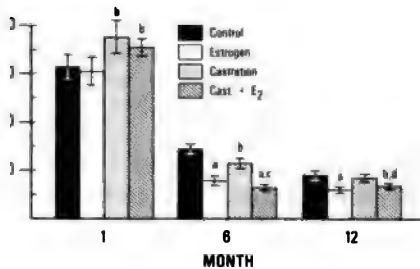


FIG. 4. *Hexosamine content of bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c)  $P < 0.01$ , or (d)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.



5. *Hexosamine specific activity.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) or (d)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.



6. *Uptake of calcium 45.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a)  $P < 0.01$ , or (b)  $P < 0.05$ . Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c)  $P < 0.01$ , or (d)  $P < 0.05$ . The same method of illustrating data is utilized in all figures.

Thus there was an increase in the uptake of active calcium, an increase in the rates of collagen and glycosaminoglycan and an increase in collagenolytic activity. Estrogen administration returned the rates to normal and decreased collagenolytic activity to somewhat below normal. Oophorectomy led to a decrease in ash content and administration to the intact animal, the oophorectomized animal returned normal.

Several interpretations are possible to explain the data in the female. Decreased sensitivity to parathyroid hormone certainly is a possible explanation. The data could also be explained by postulating an estrogen mediated increase in calcium absorption from the gut leading to a decreased need for mobilization from bone. Further work is necessary before this problem will be fully understood.

The failure of estrogen to alter the serum calcium in the male rat either indicates that the homeostatic mechanisms function better in the male or that there is a basic difference in response. The incubation studies as well as the calcium uptake indicate that estrogen given to the intact or castrated male rat causes a decrease in formation which appears to increase in magnitude until about 6 months and is still present at 12 months. The collagenolytic data demonstrates no significant change in resorption rates. This then is another difference between the male and female rat.

That estrogen has an effect on the male has been known for some time (7). Igarashi (8) demonstrated that estrogen protects the male animal against the loss of bone mineral brought about by a low calcium diet. In short term studies, Shai and Wallach (9) demonstrated once more the retardation of body and skeletal growth with an increase in skeletal mass relative to body weight brought about in male rats by estradiol. They also demonstrated a decrease in resorption and in mineral deposition as indicated by  $^{85}\text{Sr}$  studies. Finally, they demonstrated an estrogen mediated decrease in the sensitivity of male animals to the effect of exogenous calcitonin. The sex of the animal as well as its age are apparently important in determining the effect of estrogen in mediating the effect of calcitonin. Kaplan (10) showed that before puberty, the response of the two sexes was equal. Following puberty, the male decreased in sensitivity only slightly with increasing age, while females diminished rapidly. In addition and perhaps more importantly, the castrated males treated with estrogens were much less



sensitive than were the intact controls.

The end result of long term estrogen administration to the male rat is a slight but significant increase in ash content which appears to be associated with a decrease in collagen content on a per weight basis and a slight increase in hexosamine content. However, all parameters demonstrate a decrease in the rate of synthesis of bone matrix. In contrast to the data from the female rat, collagenolytic activity showed no change. These facts are difficult to reconcile because if in the face of decreased formation rate, there is an increase in bone mass, a decrease in resorption should have been measured. Perhaps the changes in collagenolytic activity which occurred were exceedingly small and resulted over a prolonged period in a decrease in resorption which could not be measured by the method utilized. It also is possible that there is a discrepancy between the mobilization rates of mineral and matrix in the estrogen treated male animal, and that in fact, the increase in ash content associated with a decrease in the organic components of matrix is reflecting this. Finally, it is possible that there is a decreased ability of the collagenolytic enzyme to actually resorb matrix, with a resultant change in resorption.

The data here do not allow one to determine the mode of action of estrogen in the male. It has been reported (11) that there is no receptor protein for estrogen in the female rat bone. It is recognized that male animals do possess receptor proteins to estrogens (12) in some tissues but up to date no reports in the literature have reported the presence of these substances in male bone cells. In addition, there is no information on a possible direct effect of estrogen on male rat bone utilizing tissue culture methods. It does, however, seem important to record the fact that male animals respond in a different fashion from females.

**Summary.** Hundred and fifty-gram male rats were divided into four groups with the first containing intact controls, the second intact animals treated with 400  $\mu$ g per 100 g body wt of 17- $\beta$ -estradiol twice a week. The animals in the third group were castrated and those in the fourth were castrated and treated with the same dosage of estrogen. Animals were sacrificed at varying periods of time

from one to 12 months. Estrogen administration caused a sustained decrease in body weight in the intact animal but did not change the body weight in castrated animals. Estrogen had no effect on either serum calcium or serum phosphorus. Estrogen administration to the intact animal caused a small but significant increase in ash content of bone. Castration caused a small decrease in this value which was still present at 12 months and estrogen administration returned the value to normal. Estrogen administration caused a decrease in total hydroxyproline content of bones of intact animals. Castration did not alter this value but estrogen administration to the castrated animal decreased the bone hydroxyproline content. Hydroxyproline incorporation rates were decreased in bones of both the intact and castrated animals. Castration did not alter the total hexosamine content of bones but estrogen administration to both the intact and castrated animals caused an increase in bone hexosamine content. Estrogen administration caused a decrease in the synthesis rate of proteoglycans in bones of both the intact and castrated animals. Estrogen administration caused a decrease in the uptake of radioactive calcium into bones of both the intact and castrated animals. There was no significant effect of estrogen on collagenolytic activity in male rat bone. It is concluded that estrogen administration to the male rat, causes changes which are different from those found in the female. There appeared to be no change in serum calcium or phosphorus values. A decreased synthesis of bone matrix and decreased uptake of radioactive calcium brought about no measurable change in the resorption of bone matrix.

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## Mechanism of the Cardiovascular Actions of Cyclocytidine (40351)

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O<sup>2</sup>,2'-cyclocytidine was synthesized to provide a useful depot form of the antineoplastic agent, arabinofuranosylcytosine (ara-C). Although one of the primary antileukemic drugs currently available (1), ara-C must be administered by frequent intermittent or continuous intravenous infusion to maintain effective plasma levels because it is rapidly inactivated by deamination in the body (2). Cyclocytidine, an anhydride analogue of ara-C, is hydrolyzed to ara-C *in vivo* and requires only once daily intravenous administration to maintain adequate ara-C plasma levels (3). In doses of 300-600 mg/m<sup>2</sup>, cyclocytidine has shown promise in the treatment of acute myelogenous leukemia in man (4). Unfortunately, cyclocytidine produces unusual side effects which limit its clinical use (5). The most pronounced undesirable side effects are sialorrhea, parotid pain and, especially, acute cardiovascular effects characterized by postural hypotension leading to syncope. Although cyclocytidine is considered overall to afford a more favorable therapeutic index than ara-C, the postural hypotension and other side effects produce sufficient patient discomfort to hamper its acceptability. The present investigation was initiated to determine the mechanism by which cyclocytidine affects function of the mammalian cardiovascular system.

**Materials and methods.** Experiments were conducted with anesthetized dogs, cats and rats. Beagle dogs of either sex (supplied by the Laboratory of Toxicology, National Cancer Institute), weighing 9-11 kg, were anesthetized with barbitol sodium (250 mg/kg) and thiopental sodium (15 mg/kg) administered intravenously. Cats of either sex, weighing 2.3-3.5 kg, were anesthetized with the barbitol-thiopental mixture given either intravenously or intraperitoneally. Male Sprague-Dawley rats, weighing 150-200 g, were anesthetized with pentobarbital sodium

(45 mg/kg) given intraperitoneally. Animals were allowed to breathe spontaneously through endotracheal tubes (dogs and cats) or through polyethylene tracheal cannulae (rats). Femoral arteries (dogs and cats) or carotid arteries (dogs and rats) were cannulated with heparin-saline filled polyethylene catheters. Systemic arterial blood pressure was measured by a Statham P23Db pressure transducer connected to a Beckman type RM oscillographic recorder. Drugs, dissolved in 0.9% sodium chloride solution, were administered into a cannulated femoral vein (dogs and cats) or jugular vein (rats) in volumes of 0.1-1 ml/kg. Blood pressure responses were measured as maximum changes in systolic pressure.

Postural hypotension was evaluated by tolerance of dogs to head-up tilt. In the tilt studies, blood pressure was measured from carotid arteries. Dogs were fastened securely to a conventional metal surgical board and one end of the board was elevated to a predetermined height for 60 sec; the angle of tilt was 20° from horizontal. The time required for restoration of systolic blood pressure to one-half of the change from pretilt values was taken as the index of tolerance to tilt.

Drugs used were cyclocytidine HCl (Drug Development Branch, Division of Cancer Treatment, National Cancer Institute), l-norepinephrine HCl (Levophed, Winthrop), tyramine HCl (Aldrich), hexamethonium chloride (City Chemical Corp.), phentolamine HCl (Regitine, Ciba), propranolol HCl (Inderal, Ayerst), guanethidine HCl (Ismelin, Ciba), desmethylinipramine HCl (Desipramine, Geigy Pharmaceuticals), and 6-hydroxydopamine HBr (Regis). All dosages were calculated as the salt forms. Statistical analyses were performed by use of the Student's *t* test, group comparisons or paired comparisons; values of *P* equal to or less than 0.05 were considered significant.

s. Cyclocytidine, in bolus doses of 100 mg/kg, increased blood pressure in dogs and rats (Fig. 1). The pressor response, which consisted of increases in both systolic and diastolic pressures, were transient and depending on the dose of cyclocytidine, they returned to preinjection values within 15 min. Responses to the highest dose of cyclocytidine often persisted for 30 min. The magnitudes of the pressor responses depended both on dosage and on route of administration. Responses to initial doses were dose-related in all three species. At 100 mg/kg, the largest dose tested, repeated injections in the same animal, however, revealed varying degrees of tachyphylaxis to the pressor effects of cyclocytidine. In separate injections of 5 mg/kg of cyclocytidine produced equivalent increases in blood pressure (Fig. 2). After a cumulative dose of 80 mg/kg, bolus injections of 80 mg/kg raised blood pressure, but the magnitude of the increase was reduced in comparison to a previous injection of 60 mg/kg. In lower doses of 25 mg/kg caused less increase in blood pressure than initial doses of 100 mg/kg (Fig. 2). After a cumulative dose of 100 or 130 mg/kg, bolus injections of 100 mg/kg of cyclocytidine elicited less pressor response than initial doses of 5 mg/kg.

Dogs were tested for tolerance to tilt before and after treatment with cumulative doses of 60 mg/kg of cyclocytidine. Before treatment, the dogs regained 50% of the pretilt blood pressure within  $17 \pm 9$  sec. After treatment with cyclocytidine, the time required for 50% recovery of carotid systolic blood pressure was  $41 \pm 9$  sec.

Experiments with a ganglionic blocker and with adrenergic drugs were conducted to locate anatomically the site of the pressor effect of cyclocytidine and to determine the mechanism by which this vasopressor drug causes postural hypotension. Administration of a ganglion blocking drug, hexamethonium (100 mg/kg), did not reduce subsequent (30 min) pressor responses to cyclocytidine in dogs (Fig. 3), cats or rats. Responses to cyclocytidine were, however, antagonized by prior administration of propranolol (10 mg/kg) and were essentially abolished by the  $\alpha$ -adrenergic receptor antagonist, phentolamine (2 mg/kg) (Fig. 3). These re-

sults suggested that cyclocytidine causes pressor responses either directly by acting upon cardiac and vascular adrenergic receptors or indirectly by promoting release of endogenous adrenergic amines. Acute administration (10–30 min before cyclocytidine) of guanethidine (2 mg/kg) completely obliterated pressor responses to cyclocytidine (Fig. 3). Desmethylinipramine (10 mg/kg) also blocked pressor responses to cyclocytidine in dogs and in rats (Fig. 3 and Table I). To establish conclusively that pressor responses to cyclocytidine result from release of norepinephrine from adrenergic neurons, rats were injected with 6-hydroxydopamine (100 mg/kg) 24 hr in advance to disrupt function of adrenergic fibers. Pressor responses to cyclocytidine and to tyramine were compared in control and in 6-hydroxydopamine-treated animals. Prior treatment with 6-hydroxydopamine nearly abolished pressor responses to tyramine and to cyclocytidine (Table II).

Pressor responses to norepinephrine and to tyramine were measured before and after acute administration of 100 mg/kg of cyclocytidine. Responses to norepinephrine were not altered by cyclocytidine treatment, but responses to tyramine were significantly reduced (Fig. 4). Pressor responses to tyramine and to cyclocytidine were not altered in animals injected 24 or 48 hr previously with 100 mg/kg of cyclocytidine.

**Discussion.** In humans, cyclocytidine causes profound changes in cardiovascular function in the usual therapeutic dose of 8–16 mg/kg (5). In this same general range of dosage, cyclocytidine causes increases in systemic blood pressure in dogs, cats and rats and, in dogs, induces cardiovascular intolerance to head-up tilt. Possible sites of cardiovascular action of cyclocytidine included baroreceptor and chemoreceptor reflex mechanisms, the central nervous system, sympathetic ganglia, adrenergic nerve terminals, adrenergic receptors and vascular smooth muscle.

The failure of hexamethonium to alter pressor responses to cyclocytidine eliminated the baroreceptor and chemoreceptor reflexes, the central nervous system, and sympathetic ganglia as potential sites of action. The pressor effects of cyclocytidine were blocked by phentolamine, indicating that it acts directly

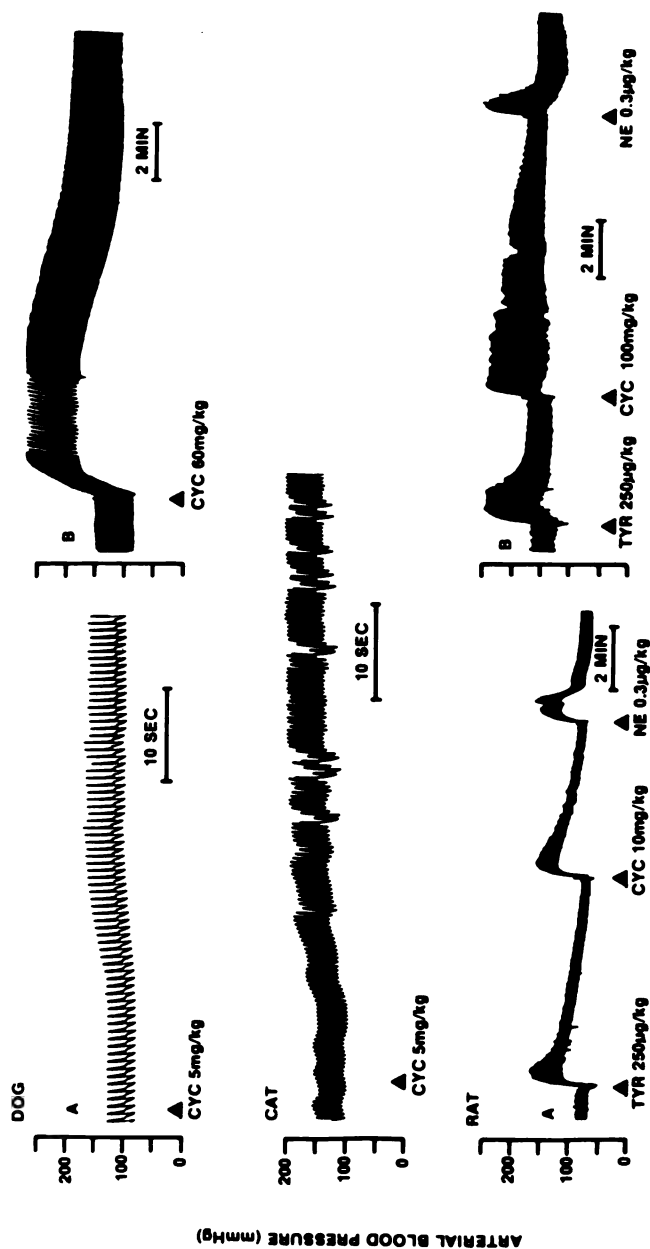


FIG. 1. Blood pressure responses to cyclocytidine (CYC) in three species. DOG. As can be seen in panel A, cyclocytidine had little effect on heart rate in dogs. Panel B shows the response to a larger initial dosage of cyclocytidine in the dog; the chart speed was increased briefly to allow counting of heart rate. CAT. The blood pressure record from the cat shows reflex slowing of the heart during the pressor response. RAT. In the rat, pressor responses to 10–100 mg/kg of cyclocytidine are equivalent to those induced by 250  $\mu$ g/kg of tyramine (TYR) or 0.3  $\mu$ g/kg of norepinephrine (NE). In the record in panel A, all pressor agents increased both diastolic and systolic pressure. In panel B, the three agents increased systolic pressure more than diastolic pressure. Blood pressure was recorded from femoral arteries in dogs and cats, from carotid arteries in rats.

or indirectly upon vascular alpha adrenergic receptors and not upon nonadrenergic vascular elements. The rapid tachyphylaxis to its pressor effects suggested that cyclocytidine could act indirectly by promoting release of norepinephrine from labile neuronal sites. Blockade of the pressor effects of cyclocytidine by guanethidine, which interferes with the adrenergic nerve uptake system and has norepinephrine antirelease properties, con-

firmed the adrenergic nerve as the site of cyclocytidine pressor effects. Blockade of cyclocytidine pressor effects by desmethylinipramine, which has little antirelease activity, could be explained by prevention of cyclocytidine entry into the adrenergic neurons (6). Finally, depletion of neuronal norepinephrine by 6-hydroxydopamine (7) demonstrated that once cyclocytidine enters adrenergic nerves, it acts by release of endogenous norepinephrine. Similar mechanisms may explain the actions of cyclocytidine on rat salivary glands, where salivation is blocked by propranolol, but not by acute sympathetic ganglionectomy (8, 9).

Cyclocytidine reduced cardiovascular tolerance to tilt, the correlate in dogs of postural hypotension in humans. The postural hypotension induced by cyclocytidine does not result from blockade of adrenergic receptors, but rather from interference with adrenergic neurons. This was shown by loss of responsiveness to tyramine, but not to norepinephrine, after acute administration of cyclocytidine. The effects of cyclocytidine on adrener-

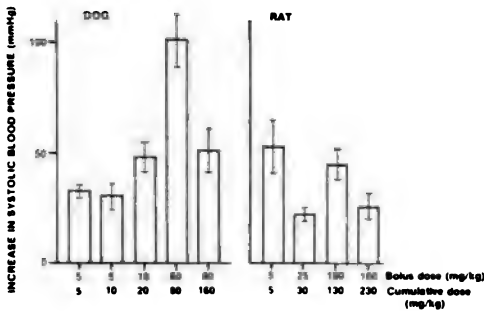


FIG. 2. Pressor responses to repeated doses of cyclocytidine in dogs ( $N = 6$ ) and rats ( $N = 6$ ). Each bar is the mean  $\pm$  SEM of the increases in systolic blood pressure.

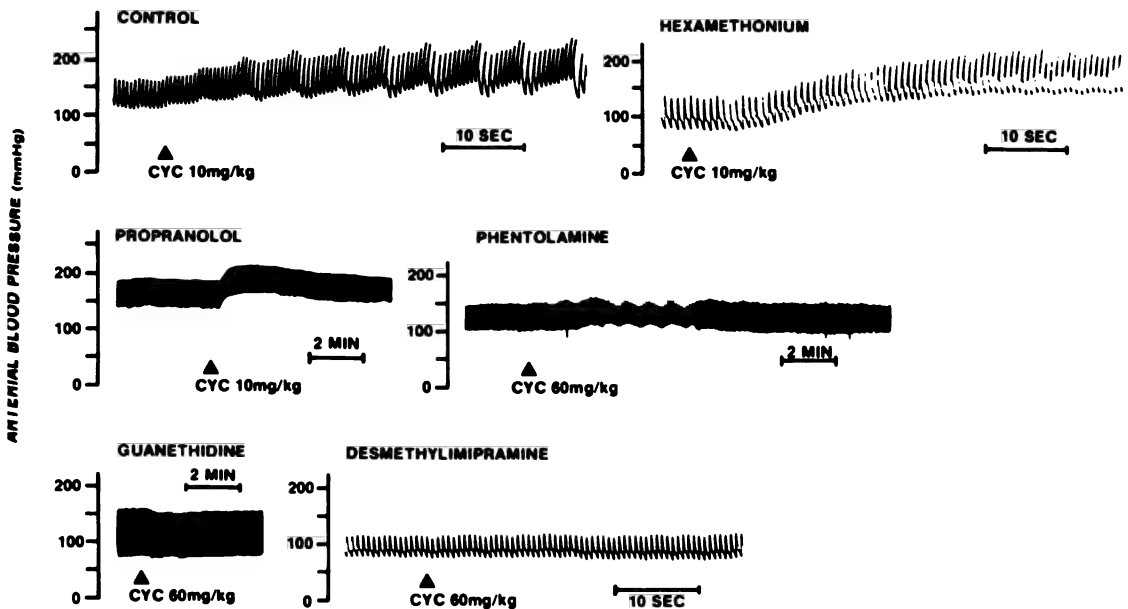


FIG. 3. Blood pressure responses to cyclocytidine (CYC) in dogs under control conditions and 10–20 min after administration of hexamethonium (20 mg/kg), propranolol (0.5 mg/kg), phentolamine (2 mg/kg), guanethidine (2 mg/kg) or desmethylinipramine (10 mg/kg). Reflex cardiac slowing is evident during the height of the control pressor response to cyclocytidine, but not in the animal treated with hexamethonium. Treatment with phentolamine, guanethidine or desmethylinipramine virtually abolished pressor effects of cyclocytidine. Blood pressure was recorded from femoral arteries.

TABLE I. EFFECTS OF DESMETHYLIMIPRAMINE (DMI) ON PRESSOR RESPONSES TO CYCLOCYTIDINE.

Species	Dose of cyclo- cytidine	Control animals		Animals treated with DMI (10 mg/kg)		P
		N	Increase in b.p. (mm Hg) <sup>a</sup>	N	Increase in b.p. (mm Hg) <sup>a</sup>	
Dog	60 mg/kg	5	101 ± 12	5	6 ± 4	<0.01
Rat	100 mg/kg	6	45 ± 7	5	16 ± 6	<0.01

<sup>a</sup> Mean ± SEM.

TABLE II. EFFECTS OF 6-HYDROXYDOPAMINE (6-OHDA) ON PRESSOR RESPONSES TO TYRAMINE AND CYCLOCYTIDINE IN RATS.

	Control animals		Animals after 6- OHDA <sup>a</sup>		P
	N	Increase in b.p. (mm Hg) <sup>b</sup>	N	Increase in b.p. (mm Hg) <sup>b</sup>	
Tyramine 200 µg/kg	6	75 ± 11	3	6 ± 3	<0.05
Cyclocytidine 5 mg/kg	6	53 ± 12	3	2 ± 2	<0.05

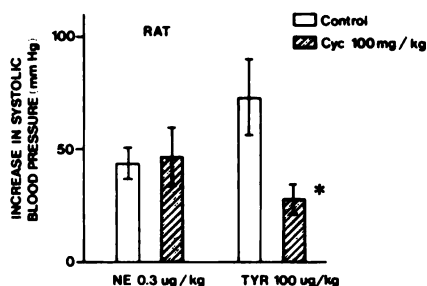
<sup>a</sup> 6-OHDA (100 mg/kg) administered 24 hr before experiment.<sup>b</sup> Mean ± SEM.

FIG. 4. Pressor responses in rats to norepinephrine (NE) and to tyramine (TYR) before (Control) and 10–30 min after administration of cyclocytidine (CYC). \*, decreased significantly from control.

gic vasoconstrictor neurons are temporary and disappear within 24 hr.

Based on these observations, we propose that acutely administered cyclocytidine enters adrenergic nerve terminals and initially promotes release of norepinephrine from a labile functional pool (10). This action causes a transient, dose-related increase in blood pressure. After the most labile pool of norepinephrine has been mobilized, the intraneuronal cyclocytidine inhibits temporarily further secretion of norepinephrine from the nerves. Responses to subsequent doses of cyclocytidine (or tyramine) are thereby inhibited. Postural hypotension could be explained by cyclocytidine-induced temporary failure of the adrenergic neuronal elements

which participate in the reflex adjustments of the cardiovascular system required for maintenance of blood pressure in response to gravitational stress.

**Summary.** The clinical usefulness of cyclocytidine, an otherwise potentially valuable antineoplastic agent, is limited because it may cause acute postural hypotension in man. In the laboratory, cyclocytidine (5–100 mg/kg) transiently increased blood pressure in anesthetized dogs, cats and rats. As the pressor responses to cyclocytidine were prevented by previous treatment with 6-hydroxydopamine or acutely by phentolamine, guanethidine and desmethylinipramine, but not by hexamethonium, adrenergic nerve terminals appear to be involved in its pressor actions. Cyclocytidine also blocked pressor responses to tyramine and caused intolerance to tilt stress in anesthetized dogs. Cyclocytidine thus appears to promote, then prevent, release of norepinephrine from adrenergic vasoconstrictor neurons.

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## Secretion of Primary Granules from Developing Human Eosinophilic Promyelocytes (40352)

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Mature eosinophils contain two types of membrane-delimited secretory granules. The primary granules are large (0.6–1.2 microns) in diameter, spherical, homogeneously dense, and are produced in the promyelocyte stage of development (1). The secondary granules produced in the myelocyte stage contain a crystalline core and represent the vast majority of the secretory granules of the mature eosinophil. Both granules stain strongly for peroxidase (1, 2). This developmental scheme has been most carefully studied in rats and rabbits (1) but is believed to occur in humans as well (2, 3).

Little is known concerning the functions of the eosinophilic promyelocyte. This is due, in part, to the very small number of these cells (less than 1%) present in normal marrow. In the present electron microscope cytochemical study we examined bone marrows of patients with disease states associated with increased numbers of eosinophilic promyelocytes along with other immature developing cells. Our evidence indicates that the contents of the primary granules of eosinophilic promyelocytes are secreted by exocytosis into the extracellular space of the marrow while the membrane surrounding the granules is retained within the cell. This secretion appears to occur simultaneously with the synthesis and production of new granules.

**Materials and methods.** Specimens consisted of bone marrow aspirates obtained from patients in the various stages of chronic myelocytic leukemia (initial diagnosis, remission, and blastic transformation) and nonleukemic states including metastatic adenocarcinoma and idiopathic thrombocytopenic purpura (ITP). These patients had increased numbers of promyelocytes and myelocytes as well as blasts in the case of CML with blastic transformation. One of the patients with

CML was studied three times; during primary diagnosis, during remission while on busulfan, and in the blast phase. The second CML patient was studied at primary diagnosis, and the third CML patient was examined during chronic phase while under treatment with busulfan. The patient with adenocarcinoma, metastatic to bone, did not have demonstrable metastatic cells on the aspirate or biopsy of the specimen examined in this study. The ITP case was newly diagnosed and on no therapy at the time the bone marrow was obtained.

The bone marrow chips were prepared for cytochemical studies according to the methods of Bainton, *et al.* (4). In brief, the marrow chips were fixed for 10 minutes in cold cacodylate-buffered 1.5% glutaraldehyde with 1% sucrose and then rinsed in cold cacodylate-buffer with 7% sucrose for 24 hr. To demonstrate myeloperoxidase activity, tissue was first soaked in the medium of Graham and Karnovsky (5) (pH 7.6) without substrate ( $H_2O_2$ ) for 10–15 min at room temperature and then incubated in the full cytochemical medium for 45 min at room temperature. Sucrose (5%) was added to all incubations. For these cytochemical studies controls consisted of  $H_2O_2$ -free media. All controls showed no demonstrable reaction product.

Following incubation, the cells were rinsed in cold 7.5% sucrose, post-fixed in cold cacodylate-buffered 1%  $OsO_4$  for 1 hr, rinsed with cold 7.5% sucrose, and soaked *en bloc* with veronal acetate buffered uranyl acetate for 30 min at room temperature. They were then rinsed in cold 7.5% sucrose, dehydrated in a graded series of ethanols and propylene oxide and embedded in Epon.

Silver to grey thin sections were cut on a Porter Blum MT2-B ultramicrotome, lightly stained with lead citrate, and examined on a

EM-100 electron microscope operated at 10 kV. Electron micrographs were taken at magnifications of 4000–15,000.

*Results.* As previously described in the prolept stage of eosinophil development, secretory granules are large, homogeneous and spherical, while the secondary granules with their characteristic crystalline inclusions appear during the myelocyte stage (Fig. 2). The rough endoplasmic reticulum of the eosinophilic promyelocyte is a contraction product for myeloperoxidase (Figs. 1, 2) and is more saccular and more extensive than its PMN promyelocyte counterpart.

Our findings provide repeated evidence that developing eosinophilic promyelocytes release the contents of their MPO positive secretory granules into the extracellular space of the marrow by exocytosis (Figs. 1–3). The electron micrographs show this degranulation also in a MPO positive RER and Golgi apparatus (Figs. 1–3).

In eosinophilic promyelocytes undergoing degranulation, there was a coalescence of individual membrane-bound secretory granules into one or more larger membrane-delimited structures each containing several granules surrounded by a single membrane (Figs. 1, inset a, and 3). Many of these structures were found to be in the vicinity of the extracellular space (Figs. 1–3). Myeloperoxidase was demonstrable within membrane-delimited granules and was released into the extracellular space (Fig. 2). The luminal surface of the membrane surrounding these multiple secretory

granules often also stain strongly for MPO (Figs. 3, 4). Several of these larger membranous structures, either devoid of any granular content or containing only a single granule, were seen within the cell cytoplasm, appearing as if they were retained in the cell following degranulation (Figs. 3, 4). This degranulation was seen in patients with CML, adenocarcinoma and ITP. We did not observe such degranulation in the numerous PMN promyelocytes nor in developing monocytes, which also contain MPO positive RER and secretory granules. Later stage eosinophilic myelocytes containing characteristic crystalline granules also did not appear to degranulate in this manner.

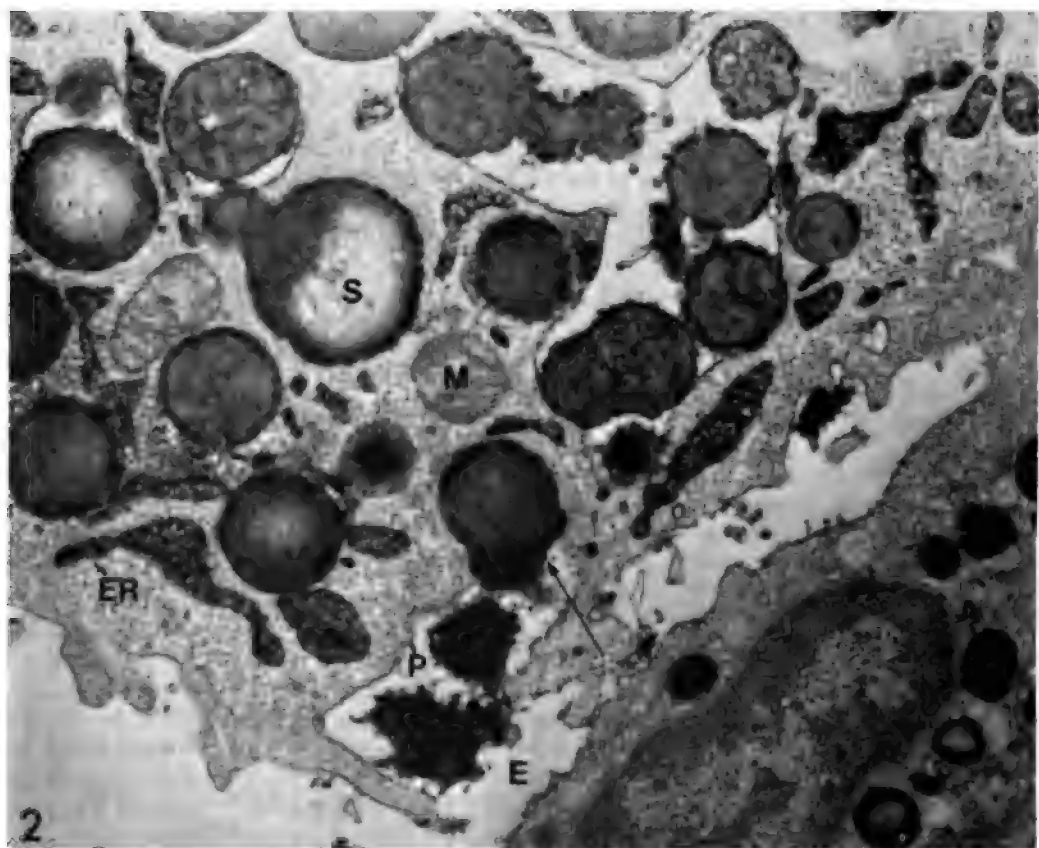
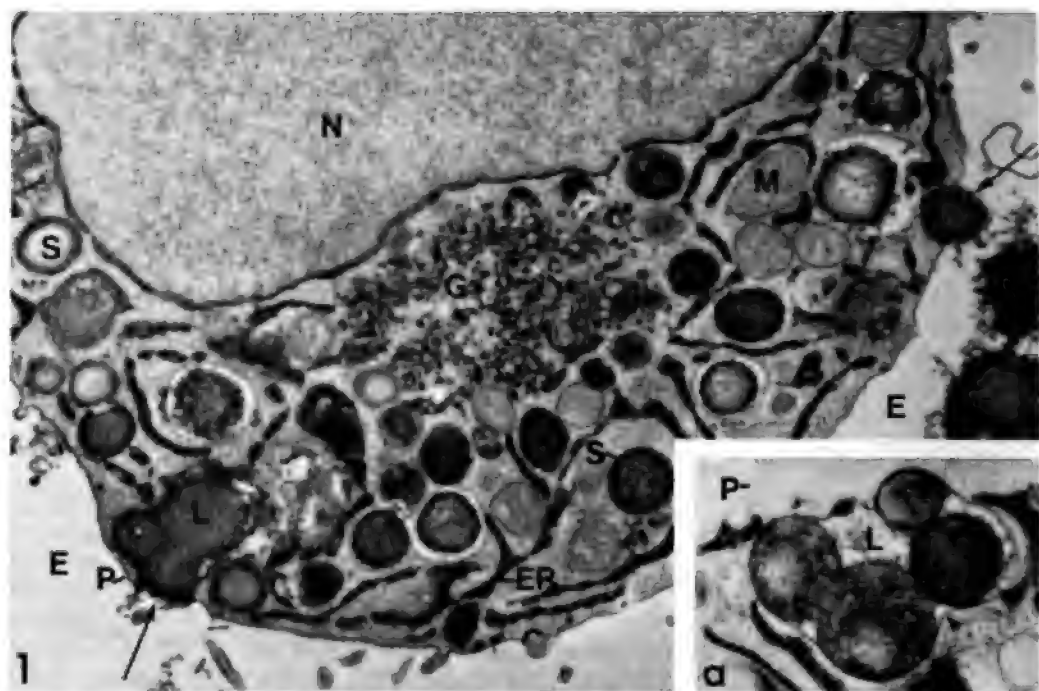
*Discussion.* The present study indicates that the homogeneous spherical primary granules formed in the eosinophilic promyelocyte can discharge their contents into the extracellular space of the marrow by a process of exocytosis, while the cell is synthesizing MPO and new granules. In a morphological study a similar phenomenon was noted in normal human marrow (6). These observations strongly suggest that secretion from eosinophilic promyelocytes consists of two steps: initial fusion of individual secretory granules to form a compound structure containing several granules surrounded by a single membrane, followed by exocytosis, the fusion of the membrane-delimited compound structure with the plasma membrane permitting access of the granule content into the extracellular space (7). This process resembles the exocytosis described as the secretory mechanism of mast cells (8).

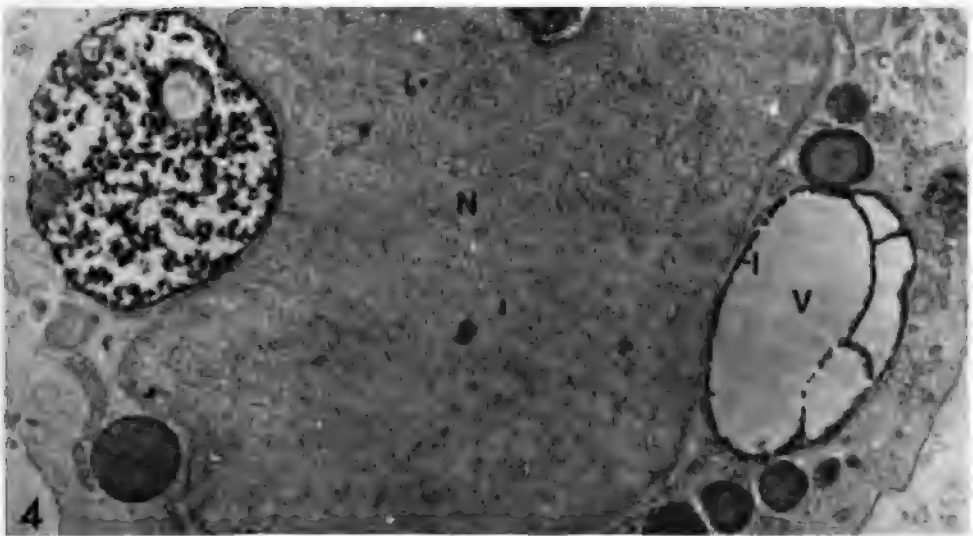
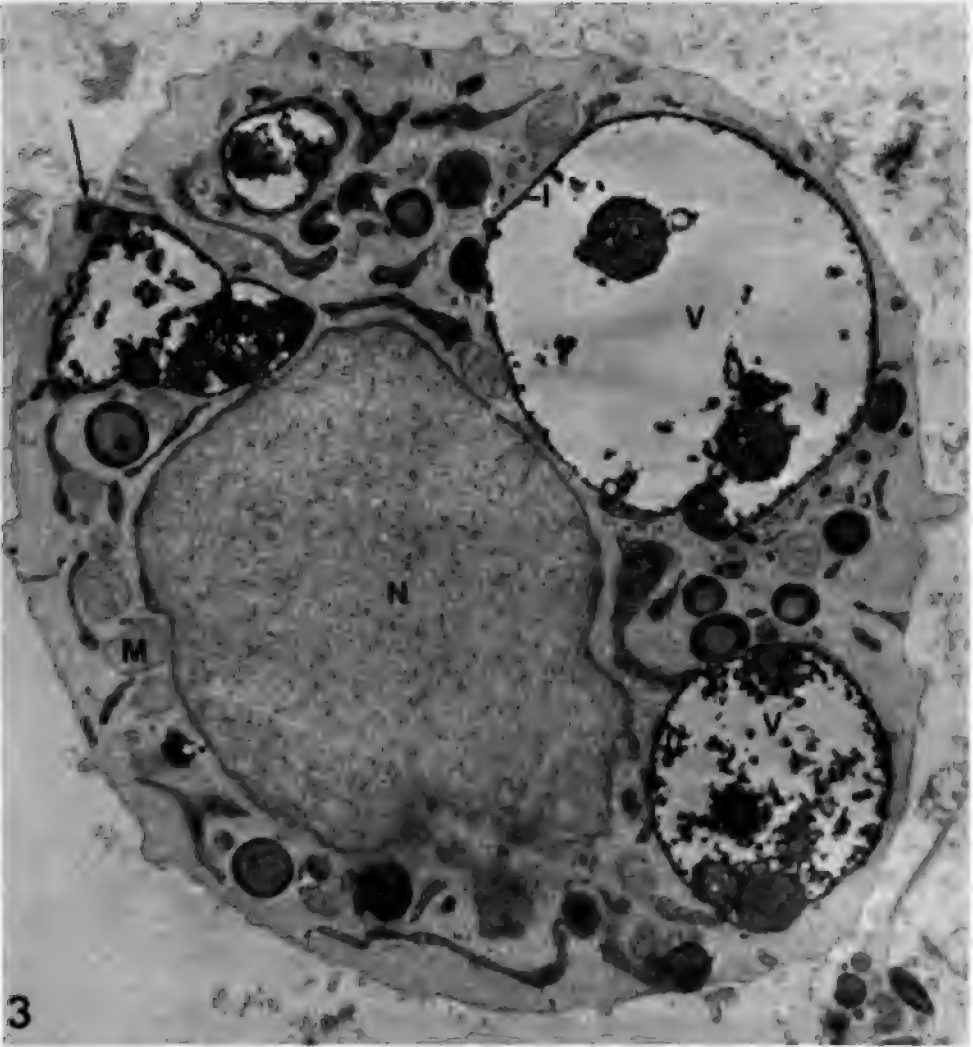
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1. Eosinophilic promyelocyte incubated for the localization of peroxidase activity. Note the large, dense, peroxidase-reactive secretory granules (S), reactive saccular endoplasmic reticulum (ER) and Golgi apparatus (G). Inset a shows secretory granules that have fused to form large membrane delimited structure (L). Larger structures appear to release their contents into the marrow extracellular space (E) by exocytosis (arrows). Plasma membrane is at P, mitochondria at M, nucleus at N.  $\times 12,000$ ; Inset a  $\times 13,200$ .

2. Higher magnification view of secretory granule release by exocytosis (arrow) from an eosinophilic promyelocyte. Plasma membrane is at P, and marrow extracellular space at E. Cytoplasm contains secretory granules, endoplasmic reticulum (ER) and mitochondria (M). Note the markedly smaller diameter of peroxidase-reactive granules (A) in an adjacent polymorphonuclear leukocyte.  $\times 24,000$ .

3 and 4. Eosinophilic promyelocytes reacted as in Fig. 1. The large vacuolar structures seen at V contain an entire granule or are entirely devoid of secretory granules (Fig. 4). Note that the inner surface of the membrane structures is reactive for peroxidase (I). An example of exocytosis is seen at arrow in Fig. 3. Nuclei are at N, mitochondria at M. Fig. 3  $\times 10,500$ ; Fig. 4  $\times 14,000$ .





In cells actively secreting materials by exocytosis, there is a considerable addition of membrane to the cell surface. A compensating endocytotic mechanism appears to retrieve surface membrane back into the cell to maintain a relatively constant surface area (9, 10). The precise nature of such retrieved membrane is not yet understood, particularly its relationship to the original secretory granule membrane. In the present study, the intraluminal surface of the large coalesced secretory granule membrane is labeled with MPO providing a potential membrane marker. It appears that this membrane delimited secretory granule only remains fused with the plasma membrane and patent to the extracellular space for a time sufficient to release the granular contents. We were able to find numerous examples of large MPO labeled cytoplasmic vacuolar structures either devoid of granules or containing very few granules. This evidence suggests that the same fragment of membrane that originally surrounded the secretory granule is retained within the cell. The subsequent fate of this membrane has not been resolved.

Developing promyelocytes of the neutrophil or monocyte series within our preparations, which also contain MPO-positive secretory granules do not show a similar exocytosis of their granules (see also 11). Therefore, we believe the events we observed in eosinophilic promyelocytes are physiological and not merely induced during aspiration of marrow or tissue preparation.

This degranulation of eosinophilic promyelocytes does not appear to be limited to any specific disease state or particular chemotherapeutic regimen. It was observed in several stages of chronic myelocytic leukemia, in adenocarcinoma and in ITP.

The significance of our observations of secretory granule release by eosinophilic promyelocytes is unclear. There is no available biochemical data on the content of these early eosinophil granules. Cytochemical studies have shown that they contain MPO, but it is not clear that these granules are biochemically identical in other respects to the later crystalloid-containing granules that are clearly a part of the lysosomal system (12-14). Previous work has demonstrated that in mature eosinophils phagocytosis is stimulated by

antigen-antibody complexes and that granules are released into the phagocytic vacuoles (14-16) but not into the extracellular space. In vitro studies have demonstrated a substance in the eosinophil granule, thought to be associated with myeloperoxidase, which causes the disruption of mast cells (17, 18). These studies hint that we may be viewing a component of an inflammatory response. Further investigation of the chemical content of these granules is clearly indicated.

**Summary.** This study indicates that the primary large homogenous dense granules of eosinophilic promyelocytes are released into the extracellular space of the marrow by exocytosis while the cell is producing new secretory granules. This process appears to occur in two steps: Initial fusion of several individual granules to form one large myeloperoxidase positive membrane-delimited body, followed by exocytotic release of the granule content. The membrane of this large secretory granule appears to be retained within the cell since empty, myeloperoxidase positive vacuolar structures remain following secretion.

The technical assistance of Ms. Dale Bloom is gratefully acknowledged.

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## Renal Tubular Secretion of Urate in Sheep<sup>1</sup> (40353)

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Renal tubular secretion of urate appears to be the rule in amphibians (1, 2), reptiles (2, 3), and birds (2, 4), where the clearance of urate exceeds the rate of glomerular filtration. In most mammals the clearance of urate is but a fraction of the filtration rate. Although the issue has been controversial, the urate of plasma probably is freely filtrable (2, 5), and there must be a net reabsorption by the tubules. In man, the urate excreted normally is only from 5 to 10% of the quantity filtered by the glomeruli (2, 6), and for some time the erroneous interpretation was that the excretion is determined simply by the balance between filtration and reabsorption. Mere comparison of the urate clearance with that of inulin does not provide any evidence for renal tubular secretion of urate in most mammals.

The data in the present paper indicate that a net renal tubular secretion of urate normally occurs in sheep, as shown by the ratio of the clearances of urate and inulin.

**Material and methods.** Renal clearances of inulin and urate were measured in seven ewes; two were normal nonpregnant sheep, three were in the last weeks of normal pregnancies, and two, near term, were moribund with ovine toxemia of pregnancy. The five normal ewes stood during the procedure and the two toxemic sheep lay on their sides.

Inulin was injected as a priming dose and given by constant infusion in 5% dextrose at 4 ml/min, in amounts calculated to maintain the level in plasma at about 30 mg/100 ml. An hour was allowed for equilibration and

the establishment of nearly constant rates of urinary flow before beginning the three clearance periods. Urine was obtained by Foley catheter, with two rinses of the bladder, each with 30 ml of water and about 30 ml of air. Venous blood samples were taken at the mid-point minus 5 min between collections of urine. Serum was used for the analysis. In the normal sheep the clearance periods were from 20 to 30 min; in the two oliguric sick animals the periods were from 1 to 2 hr.

In preliminary experiments, we found that the sera of blood samples from sheep (controls) had high blank readings in the method of Roe, Epstein, and Goldstein (7) for the measurement of inulin, presumably because of endogenous fructose. In each measurement of serum inulin we corrected for the blank for the particular animal, on the unproved assumption that the blank did not change significantly during the course of the observations.

We also found that urate in serum often was undetectable by Folin's (8) indirect method, although urinary concentrations were so high as to necessitate dilutions of from 50 to 100 times for analysis. We considered the possibility that some complex of urate in serum is precipitated by tungstic acid, or that there is some inhibitor of the chromogenic reaction in serum. Folin's indirect method, however, gave readable levels of color in sera that did not react in the direct method. In two experiments the indirect method gave nearly identical values in ultrafiltrates and in tungstic acid filtrates of sera. We then used both the direct and indirect methods for: (a) Diluted urines and tungstic acid filtrates of sera; (b) the same, previously treated with uricase; (c) the same, to which known amounts of urate had been added, (d) analyzed as such, and (e) analyzed after treatment with uricase. Water and reagent blanks

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were carried through all procedures. When uricase was used, each of 2 ml of water, 2 ml of diluted urine, and 5 ml of serum were placed in 50 ml volumetric flasks and 5 ml of borate buffer at pH 9.2, 50 mg of uricase, and 10 ml of distilled water were added to each. After incubation at 45° for 2 hr, 1 ml of 10% sodium tungstate was added to each and then 8 ml of N/12 sulfuric acid, slowly and with constant mixing. The preparations were diluted to volume, mixed, and filtered after standing for 10 minutes. Folin's methods were then applied to the filtrates. All readings were made with a Coleman junior spectrophotometer.

Although Folin's indirect method always gave readable color in filtrates of serum, we modified the method by precipitating the silver salt of urate from five times the usual volume of filtrate. That is, in the analysis of human serum, Folin used 5 ml of filtrate, representing 0.5 ml of serum; for measurements in ovine serum we used 25 ml of filtrate, representing 2.5 ml of serum, because of the low concentrations that ranged from 0.1 to 0.2 mg/100 ml in the normal animals and were 0.32 and 0.74 in those with toxemia.

The urate clearances that we report are based upon serum urate as measured by the modified indirect method, and urinary urate as measured by the direct method.

**Results. Chromogens in urine and serum.** The urinary substance(s) that developed color in both direct and indirect methods for urate was really urate, as indicated by the destruction of from 96.4 to 100% of the chromogen by uricase. Uricase destroyed virtually all of the chromogen in serum. Moreover, the indirect method is alleged to be specific for urate (8), and the clearances were calculated from indirect measurements of urate in serum.

*Recovery of urate added to urine and serum.*

Urinary and serum samples were mixed with equal volumes of an aqueous solution containing 0.02 mg/ml of uric acid. After standing, one aliquot was treated with uricase, as described above. The treated and untreated aliquots were then carried through the direct and indirect procedures. The recovery of urate varied from 93.5 to 108% (average, 99.6%) in the direct method and from 88 to 104% (average 96.0%) in the indirect method.

The amount of urate added to diluted urine was well within the range of the endogenous levels measured. Unfortunately, we added far too much to serum and the recoveries do not validate the estimates of endogenous levels, even though we used five times the usual volume of filtrate in the measurements.

**Inulin clearances.** As shown in Table I, the mean inulin clearances in the three normal pregnant ewes were 101, 98, and 115 ml/min; in the two normal nonpregnant sheep they were 73 and 70 ml/min. The clearances are well within the range that Parry and Taylor (9) observed and collected from the literature. The apparent clearances in the two sick animals varied greatly from period to period, cannot be averaged, and clearly are unreliable, perhaps because of varying delivery of urine from the ureters to the bladder in the oliguric ewes. Nevertheless, the ratios of urate/inulin clearances seem valid; we report them because they are consistent with the findings in the normal sheep, despite the profound depression in renal function.

**Urate clearances.** In every clearance period in every animal, pregnant or not, normal or sick, the urate clearance was greater than the simultaneous inulin clearance by from 52 to 290%, with consistent ratios from period to period in each animal. The ratio of urate clearance/inulin clearance averaged 2.46 for all observations.

Folin (8) wrote that the direct method,

TABLE I. RENAL CLEARANCES OF INULIN AND URATE IN SHEEP

Averages of three clearance periods						
Status	Weight, kg	Urinary vol- ume, ml/min	Serum urate mg/100 ml	Clearances, ml/min		Clearance ratio Urate/inulin
				Inulin	Urate	
Pregnant	53.6	5.48	0.17	101	284	2.8
Pregnant	63.2	3.21	0.11	98	265	2.7
Pregnant	64.2	6.90	0.14	115	194	1.7
Non pregnant	59.1	7.50	0.20	73	128	1.8
Non pregnant	39.6	4.04	0.20	70	196	2.8
Toxicemic	62.6	1.10-2.34	0.32	13-62	38-180	2.9-3.9
Toxicemic	77.3	0.67-0.88	0.74	0.1-0.9	0.2-2.0	2.0-2.3



which we used for urine, gives urinary values that "were nearly always from 5% to over 10% higher than those obtained by the indirect method applied to diluted urines." Thus, the clearances that we report are too high by that range of percentages, but it is obvious that the clearance of urate is so much greater than that of inulin that an error of 10% is of little significance.

Thus, on the average, more than half of the excreted urate must have been secreted by the tubules. Nearly all, or all, may have been if the filtered urate had been reabsorbed, as it is in man.

**Discussion.** Renal tubular secretion of urate appears to be a phylogenetically ancient process that may have persisted in many, if not all, higher animals despite the later superimposition of tubular reabsorption of the substance. The current concept (2, 10) is that there are four processes involved in the excretion of urate by man and probably by other mammals. (a) Glomerular filtration of urate, (b) tubular reabsorption of nearly all of the filtered urate, (c) tubular secretion of urate, and (d) tubular reabsorption of some of the secreted urate (postsecretory reabsorption).

Praetorius and Kirk (11) described an anomalous young man with marked hypouricemia whose renal clearance of urate exceeded that of inulin; he, therefore, must have had renal tubular secretion of urate. Gutman, Yü, and Berger (12) demonstrated ratios of excreted urate/filtered urate greater than 1.0 in gouty and normal men who were loaded with a potent uricosuric agent (sulfipyrazone), thus clearly showing tubular secretion of urate in that circumstance. They suggested that in man perhaps all of the filtered urate normally is reabsorbed and whatever is excreted reaches the urine by tubular secretion.

The dalmatian coach hound is exception in that the renal clearances of urate and creatinine are virtually identical (13), or the urate clearance is the greater of the two (14).

Poulsen and Praetorius (15) observed that in the rabbit the ratio of endogenous urate to creatinine clearance averaged 0.40, with a single maximal value of 0.75. They infused urate to increase its concentrations in plasma of from 2 to 5  $\mu\text{g}/\text{ml}$  up to from 10 to 30  $\mu\text{g}/\text{ml}$ , and found that the ratio of  $C_{\text{Ur}}/C_{\text{Cr}}$  in-

creased to an average of 1.77 (range of 1.25–3.0 in 32 clearance periods). That is, the infusion of urate had stimulated a net tubular secretion of the substance.

Fanelli *et al.* (16) studied seven species of Old World monkeys and found that the urate clearance exceeded the inulin clearance in all but the bushbaby. In 12 species of New World monkeys, in the gibbon, and in the chimpanzee, the urate clearance was less than the inulin clearance in all but the red howler. Because of the low concentrations of urate in plasma, Fanelli *et al.* loaded the animals with urate "when indicated", and whether any animal showed a net tubular secretion of urate in the absence of loading is not specified. Net tubular secretion of urate has been observed in goats (2), calves (2), pigs (19), and guinea pigs (2, 18), but urate had been infused to raise its level in plasma. Mudge, McAlary, and Berndt (18), in their study of guinea pigs, usually infused urate but did find net tubular secretion of urate in four animals whose endogenous clearances were measured. Simmonds, Cameron, and Potter (19) recently reported that the renal clearance of endogenous urate exceeds that of inulin in pigs. Thus, the sheep is not unique in having a net tubular secretion of urate.

**Summary.** The simultaneous renal clearances of endogenous urate and of inulin were measured in five normal ewes, three pregnant and two not, and in two sheep moribund with ovine toxemia of pregnancy. The urate clearance exceeded the inulin clearance in every period in each sheep, with the ratio ranging from 1.7 to 3.2 and averaging 2.46.

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Effects of Tetraethylammonium and Manganese on Mesenteric Vasoconstrictor Escape<sup>1</sup> (40354)

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The *in vitro* contractile response of cat mesenteric arterial rings to norepinephrine (NE) is frequently a phasic contraction which reaches a peak in 1-2 min and then fades ("escapes") despite the continuing presence of NE. An earlier study from this laboratory (1) showed that the phasic contraction could be converted to a tonic (nonescaping) response by (a) reducing the external calcium concentration, (b) pretreating with verapamil or (c) depolarizing the vessel by increasing the external potassium-ion concentration.

These observations suggested that the phasic response of the cat mesenteric artery might be associated with calcium-dependent action potentials ("calcium spikes"). If this were so, tetraethylammonium which augments calcium spikes should enhance the phasic contraction whereas manganese, which inhibits calcium-spikes, should diminish it (2).

**Methods.** Male cats weighing 3-5 kg were anesthetized with intraperitoneal sodium pentobarbital 40 mg/kg. The superior mesenteric artery was dissected free of connective tissue *in situ* and then removed. Rings 2-5 mm long and about 1 mm in diameter were cut from the artery and placed in a physiological salt solution (PSS) containing (in mM): NaCl 123, KCl 5, CaCl<sub>2</sub> 1.6, MgCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 25, CaNa<sub>2</sub>EDTA 0.026, ascorbic acid 0.01 and glucose 11.1. This solution, referred to as regular PSS, was aerated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>; its pH was 7.4. The arterial ring was mounted between a stationary stainless steel rod and a Statham UC-2 strain gauge connected to a Hewlett-Packard 7700 recorder. The mounted ring was immersed in a 20 ml bath containing PSS solution at 37° and was stretched during the equilibration period to maintain a force of approximately 500 dynes. Every 20 min, NE (Levophed, Winthrop Laboratories) was

added to the bath and washed out after 5 min. Two more washes were performed before the next NE dose was applied. Two to four hours were required to achieve stable responses. The effects of tetraethylammonium (TEA) 0.06-10.0 mM were studied by adding TEA chloride (J. T. Baker Chemical Company) to the bath 5 min before each NE test dose. The effects of higher TEA concentrations were studied by substituting equimolar amounts of NaCl by TEA Cl.

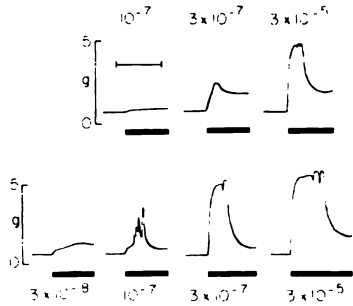
Some experiments were performed after depolarizing the vessel rings by substituting the regular PSS in the bath with a depolarizing solution containing (mM) KCl 3, KHCO<sub>3</sub> 25, K<sub>2</sub>SO<sub>4</sub> 86, CaCl<sub>2</sub> 1.6, MgCl<sub>2</sub> 1.2, CaNa<sub>2</sub>EDTA 0.026, ascorbic acid 0.01 and glucose 1.1.

Statistical significance was determined by Student's *t* test for paired comparisons.

**Results. Effects of TEA alone.** Concentrations of TEA below 40 mM had no effect on resting tension in any artery. Higher concentrations induced weak tonic contractions in arteries from six of seven animals. The threshold was between 40 to 80 mM in four arteries and between 80 to 120 mM in two. The TEA contractions never exceeded 15% of the maximum NE response.

**Effects of TEA on the NE response.** Figure 1 shows the responses of a mesenteric arterial ring to increasing doses of NE and the effects of 2 mM TEA. Note that before TEA, NE 10<sup>-7</sup> g/ml, a dose close to threshold, produced a tonic contraction of 200 mg. The same NE dose, after pretreatment with TEA, caused a series of phasic contractions with a peak force of 2.8 g after 2 min. Force then declined, despite the continuing presence of NE, to a steady-state force of 300 mg. The figure also shows that TEA enhances the initial component of phasic contractions but not the steady-state response. Additionally, it is seen that the maximum phasic response to NE (3 × 10<sup>-5</sup> g/ml) was 4 g before TEA and was

<sup>1</sup> Supported by USPHS Grant No. HL 18199.



1. Contractile responses of cat mesenteric arterioles to NE before and after TEA. Numbers indicate concentrations (g/ml). Upper traces: before TEA, lower traces: after pretreatment with 2 mM TEA. Time bars 5 min. The black bar below each trace indicates period during which NE remained in contact with vessel.

increased to 5 g after TEA.

Twenty-eight percent of mesenteric rings did not show phasic contractions but showed sustained tonic responses to all doses of NE. TEA converted these into phasic contractions that attained higher peaks but then escaped to baseline levels of force lower than those seen in the absence of TEA.

The differential effects of TEA on the phasic and steady-state components of the response were examined quantitatively in arterial rings from 12 cats. An approximately ED<sub>50</sub> dose of NE was determined for each cat and the effects of pretreating the vessel 15 min with varying doses of TEA, over a range 0.06–120 mM, were measured (Fig. 2). TEA caused a dose-dependent potentiation of the initial component of the NE response. In contrast, TEA inhibited the steady-state response. These effects were maximal at a TEA concentration of 20 mM.

**Effects of TEA on NE response in calcium-free solution.** After 20 min exposure to calcium-free PSS solution, the response to NE was greatly reduced and its phasic character lost. The response to a maximal NE dose of  $4 \pm 0.4$  g ( $n = 4$ ) in regular PSS solution was reduced to  $0.1 \pm 0.05$  g ( $n = 4$ ) in calcium-free

solution. Pretreatment with TEA had no significant effect ( $P > 0.1$ ) on the NE contractions of arterial rings in calcium-free PSS. An example is shown in Fig. 3.

**Effects of TEA on NE response in depolarizing PSS.** When mesenteric arterial rings

were transferred to depolarizing PSS a substantial contracture developed. The addition of NE produced a tonic increase in this contracture. Pretreatment of the vessel with 2 mM TEA had no significant effect ( $P > 0.1$ ) on the response either to depolarizing PSS or the subsequent addition of NE ( $n = 5$ ). An example is shown in Fig. 4.

**Effects of Manganese on the NE response (four cats).** Segments which gave phasic responses to NE were exposed to MnCl<sub>2</sub> for 10 min prior to and during the addition of NE. Manganese concentrations in the range 0.04–0.1 mM reduced the phasic component of the NE response whereas manganese concentrations in the range 0.1–0.3 mM abolished them. An example is shown in Fig. 5.

**Discussion.** A number of investigators (2–6)

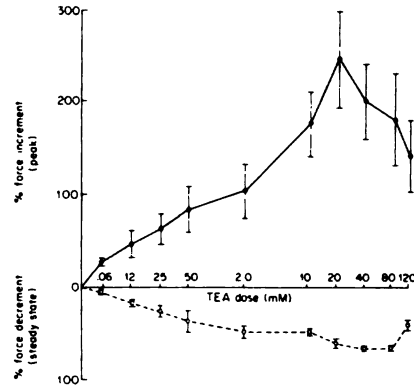


FIG. 2. Effect of pretreatment with various concentrations of TEA on peak (●—●) and steady-state (○—○) responses to approximately ED<sub>50</sub> NE doses. TEA concentrations between 0.06 and 2.0 mM were tested in five cats. TEA concentrations between 10 and 20 mM were tested in another group of seven cats. The abscissa scale is logarithmic. The effects are shown as percent changes from control. Values are means  $\pm$  SE.

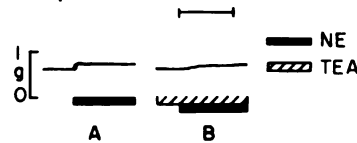


FIG. 3. Failure of TEA to alter NE response in calcium-free solution. A—before TEA, NE  $3 \times 10^{-7}$  g/ml produced a tonic contracture of only 100 mg. B—after 10 min pretreatment with 2 mM TEA the NE response was unchanged. Contrast this with the effect of TEA on the same dose of NE in regular PSS (Fig. 1). Time bar = 5 min.

have previously shown that TEA augments the responses of isolated arterial strips to a variety of agonists. Kalsner (5) suggested that the augmentation was due to enhanced calcium mobilization. Haeusler and Thorens (6) obtained direct evidence for this by showing that 10 mM TEA enhanced calcium influx in isolated rabbit pulmonary arteries. They also showed that 10–100 mM TEA induced a dose-dependent depolarization of pulmonary arterial smooth muscle.

All previous studies of TEA potentiation of arterial vasoconstrictor responses have used preparations which show only tonic responses to agonists. The present investigation is the first to examine the effects of TEA and manganese on a vessel which commonly shows a striking "fade" or "escape" of the mechanical response during continuing NE exposure. The principal findings were that (a) TEA potentiated the initial component of the NE response but not the steady-state response; (b) TEA potentiation did not occur in completely depolarized vessels or in vessels exposed to calcium-free solution; (3) manganese inhibited the initial component but not the steady-state component of the NE response. These observations suggest that the steady-state response is dependent upon a different excitation or excitation-contraction coupling mechanism than the initial portion of the response. In a previous paper (1) it was reported that NE-induced phasic contractions of cat mesenteric arteries were blocked by pretreatment with calcium-free solution, verapamil or potassium-rich solutions and it was suggested that the phasic response might be associated with calcium-spikes. The present observations support this view. TEA augments calcium-spikes by blocking the late

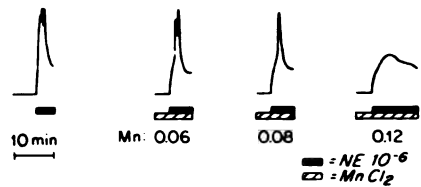


FIG. 5. Effects of  $\text{MnCl}_2$  (numbers indicate mM) on the mesenteric arterial response to NE.

potential-dependent increase in potassium conductance which limits the degree of depolarization which can be induced by calcium influx (2). Thus, contractions dependent upon calcium spikes should be potentiated by TEA. This was clearly the case for the initial component of the mesenteric NE response (Figs. 1, 3). In contrast, the steady-state response was inhibited by TEA. The mechanism of this inhibitory effect is not revealed by these experiments, but the very absence of potentiation indicates that this part of the response is not based on calcium-spikes and may be dependent upon pharmacomechanical coupling. The fact that the mesenteric artery will respond to NE when completely depolarized and that TEA does not alter the response supports this view.

Manganese is known to block calcium-spikes in many tissues (2) and in the low concentrations used in our experiments, it blocked the phasic component of the NE response but not the steady-state response.

The effects of TEA and manganese, therefore, appear to support the hypothesis that mesenteric vasoconstrictor escape may be due to the inability of mesenteric arterial smooth muscle to sustain action potentials for more than a minute or two following NE administration.

**Summary.** Norepinephrine (NE) induced either phasic or tonic contractions in isolated rings of cat mesenteric arteries. Tetraethylammonium (TEA), 0.6–120 mM enhanced the peak contractile response to NE but reduced the steady-state response. Manganese, 0.06–0.12 mM, inhibited the peak NE response with no effect on steady-state force development. TEA-potentiation was maximal at 2–20 mM. No potentiation occurred in calcium-free solutions or when the vessel was depolarized by high external potassium concentrations. These observations provide

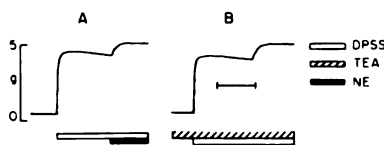


FIG. 4. Effects of TEA on the NE response of a mesenteric arterial ring treated with depolarizing solution (DPSS). Note that DPSS produces a large tonic contracture which is augmented by NE. The response before TEA (A) does not differ significantly from the response after 10 min pretreatment with 2 mM TEA (B). Time bar = 5 min.

circumstantial evidence that mesenteric vasoconstriction may be associated with "calcium-spike" activity and that vasoconstrictor escape may be due to fading of this activity.

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## Mammary Arterial and Venous Concentrations of Serum Insulin in Lactating Dairy Cows<sup>1</sup> (40355)

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Insulin is essential for lactation (1). Concentrations of insulin in plasma increase with lactogenesis in rats (2) and increase in sera of cows as lactation progresses (3, 4). Furthermore, blood collected from cows immediately after milking contained greater concentrations of insulin than blood collected 2-4 hr before or 1 hr after milking (3). Presumably for insulin to affect mammary tissue it must be removed from blood and bound to mammary cells. Indeed *in vitro* studies of mammary epithelial cells from lactating mice showed that <sup>125</sup>I-insulin binds to membrane receptors (5). The primary objective of the present study was to measure arteriovenous (A-V) differences in serum insulin across the mammary glands of cows around milking.

**Materials and methods.** Twelve Holstein cows, six lactating 5-12 weeks and six lactating 37-57 weeks were used. Cows were maintained in stanchions and fed a ration of 18 kg of corn silage, 4.5 kg alfalfa-grass hay and 1 kg of grain concentrate per 2.5 kg of milk produced. Water was provided *ad libitum*.

One cannula was implanted surgically into an external pudendal artery and another into a subcutaneous abdominal mammary vein as previously described (6). Experiments commenced 3-5 days after surgery when milk yields approximated pre-surgery quantities.

Cannulas were flushed approximately 2 hr before each experiment and blood was collected and discarded every 15 min to accustom cows to sampling. Arterial and venous samples of blood were collected simultaneously on three consecutive afternoons at 30, 25, 20, 15, 10, 8, 6, 4, 2 and 0 min before milking and at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20,

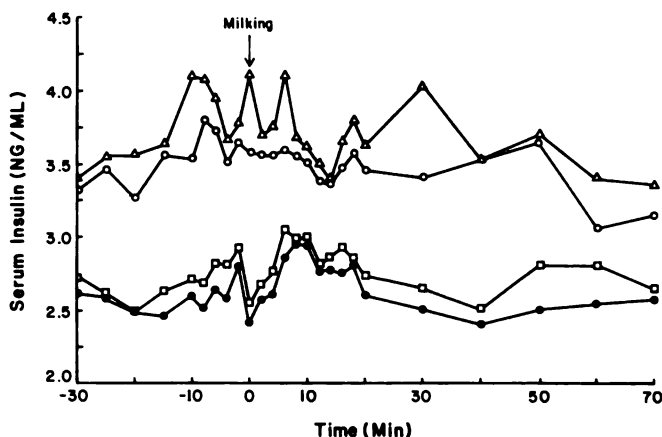
30, 40, 50, and 60 min after milking. At time 0 the mammary glands were washed for 20-30 sec and then milked for 3-5 min with a mechanical milking machine. Milking occurred at approximately 1500 hr each day. Blood was stored at  $\approx 25^\circ$  for 2 hr, at  $5^\circ$  for 24-36 hr and then centrifuged at 2500g for 15 min. Sera were stored frozen at  $-20^\circ$  prior to assay for insulin. Radioimmunoassay for insulin was as described previously (3, 7). Standard bovine insulin (Lot No. 795372; 24.2 units/mg) was provided by Eli Lilly and Co. (Indianapolis, IN). Hormone concentrations were determined in duplicate in each serum sample and accepted when agreement between duplicates was within  $\pm 5\%$ . Within each time of sampling insulin concentrations were averaged across the three experimental replicates (days) for each cow. These values were used in a split-plot analysis of variance (8).

**Results.** Insulin in arterial and venous sera of cows lactating 5-12 weeks averaged ( $\pm$ SE) overall throughout the experiment  $2.7 \pm 0.4$  and  $2.6 \pm 0.4$  ng/ml, respectively (Fig. 1). In cows lactating 37-57 weeks insulin averaged  $3.6 \pm 0.2$  and  $3.4 \pm 0.1$  ng/ml, respectively. Insulin was greater in arterial ( $P \approx 0.08$ ) and venous ( $P \approx 0.09$ ) sera of cows lactating 37-57 weeks as compared with insulin in cows lactating 5-12 weeks. Stimuli associated with milking did not affect concentrations of serum insulin in either early or late lactating cows.

For the 30 min before milking, mammary arterial concentrations of insulin were  $0.13 \pm 0.04$  ng/ml greater ( $P < 0.05$ ) than venous concentrations in cows 5-12 weeks postpartum. In cows lactating 37-57 weeks the A-V difference was  $0.22 \pm 0.08$  ng/ml, but this difference was not significant ( $P > 0.05$ ). During the 20 min beginning at milking arterial concentrations of insulin were greater ( $P < 0.05$ ) than venous concentrations in

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1. Insulin concentrations in serum samples from early (5–12 weeks postpartum) lactating (arterial  $\Delta$ ; venous  $\bullet$ ) and late (37 to 57 weeks postpartum) lactating (arterial  $\triangle$ ; venous  $\circ$ ) cows during and after milking which began at 0 min and lasted 3–5 min. Each point is the mean serum insulin concentration from three replicates in each of six cows. Pooled SE among early lactating cows were 0.4 and 0.4 ng/arterial and venous samples, respectively. Among late lactating cows the pooled SE were 0.2 for arterial and 0.1 ng/ml for venous samples, respectively.

( $0.12 \pm 0.03$  ng/ml) and late ( $0.17 \pm 0.19$  ng/ml) lactating cows. Between 30 and 70 min postmilking, the A-V differences in concentrations of insulin in cows 5–12 weeks postpartum ( $0.20 \pm 0.09$  ng/ml) or 37–57 weeks postpartum ( $0.23 \pm 0.14$  ng/ml) were not significant ( $P > 0.05$ ). Stage of lactation did not affect ( $P > 0.05$ ) A-V differences in insulin.

To investigate mammary uptake of insulin, mammary blood flow (MBF) was calculated using the equation of Kronfeld *et al.* (9) in which  $MBF = 1.0 + 0.42 \times$ , where  $\times$  is daily milk yield. Daily milk yields averaged 22.9 kg in early lactating cows and 13 kg in late lactating cows. Thus, MBF were estimated to be 6.5 and 6.6 liters/min. A-V differences averaged 0.15 and 0.21 ng/ml for cows in early and late lactation, respectively. Theoretical mammary uptakes of insulin (calculated by multiplying MBF by A-V differences) were 1.4  $\mu$ g/min in early and late lactating cows, respectively.

**Discussion.** Serum insulin concentrations in early and late lactating cows remained remarkably constant in the 25 samples of sera collected between 30 min prior to milking through 70 min after milking. This is in contrast with the previous report of Koski and Tucker (3) who observed greater concentrations of insulin in sera collected 15 min of milking compared with sera

collected 2–4 hr before or 1 hr after milking. The cause of the discrepancy is unknown, but may be associated with differences in feeding schedule relative to milking. In any event the present study strongly suggests that milking does not cause an acute increase in concentrations of insulin in cows.

Insulin A-V differences across the mammary gland were positive and remarkably similar regardless of time relative to milking or stage of lactation (and milk yield). Maintenance of positive A-V differences in serum insulin during the interval from 30 min before through 70 min after milking suggests the possibility of continuous mammary uptake of the hormone from arterial blood. We speculate this uptake of insulin is probably essential for regulation of uptake of metabolites and maintenance of lactation (1).

The greater concentrations of serum insulin observed in late lactating cows producing 43% less milk per day compared with early lactating cows agrees with previous reports (3, 4). In dairy cows, serum insulin concentrations are negatively correlated with milk yield (3), and greater concentrations of serum insulin in beef cattle, compared with dairy cattle, may be associated with their lower rate of milk production (10). Also, administration of insulin suppresses milk yields in cattle unless exogenous glucose is supplied simultaneously (11). Since numbers of mammary



secretory cells decrease with advancing lactation or decreasing milk yields (12) while total uptake of insulin remained essentially constant, the uptake of insulin per mammary cell theoretically increases with advancing lactation. If and how the theoretically greater uptakes of insulin per mammary cell are associated with suppression of milk synthesis remains to be determined. On the other hand, serum insulin increases as feed intake increases relative to maintenance requirements (13). In our study early and late lactating cows were fed the same rations. Most likely the late lactating cows were fed in excess of requirements for milk yield. Thus, the increased serum insulin during late lactation may be related to diet and only coincidentally related to milk production.

**Summary.** Insulin averaged 2.6 ng/ml in mammary arterial and venous sera collected from 30 min before to 70 min after milking of cows lactating 5–12 weeks. During the same period in cows lactating 37–57 weeks insulin increased to 3.5 ng/ml. Milking did not affect insulin concentrations during early or late lactation. Arteriovenous (A-V) differences averaged 0.17, 0.14 and 0.22 ng/ml for 30 min before, 0–20 min after and 30–70 min after milking. Stage of lactation (and yield of milk) did not affect A-V differences. Mammary uptakes of insulin averaged 1.6 and 1.4  $\mu$ g/min in early and late lactating cows, respectively. Maintenance of uptakes of insulin

may be associated with uptake of metabolites essential for maintenance of lactation.

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## Pituitary Response to TRH and LHRH in Spontaneously Hypertensive Rats (40356)

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The isolation from Wistar rats of a strain with spontaneous hypertension has provided investigators with an excellent experimental model for studying essential hypertension in man. Like man in the early stages of essential hypertension (2), the young, spontaneously hypertensive rat (SHR) responds to stress with exaggerated blood pressure and heart rate rises (3). It has been proposed that the cardiovascular system of the SHR is extremely sensitive to increased neurohormonal stimulation due to exaggerated hypothalamic defense activity (4). The SHR has been noted to have a larger pituitary, thyroid and adrenal glands and to have intensified activity of the hypothalamic-adrenal and ACTH-corticoid axis in comparison to Wistar controls (1, 5). In this study we have examined the luteinizing hormone-releasing hormone (LHRH) and the thyrotropin (TSH) and prolactin (PRL) response to thyrotropin releasing hormone (TRH) in SHR and normotensive control rats in an attempt to determine if the pituitary response to these releasing hormones is altered in the SHR.

**Materials and methods.** Fourteen male and 14 normotensive Wistar Kyoto rats weighing 180–225 g were individually caged and maintained at  $(27 \pm 2^\circ)$  on a 14:10 dark cycle. The animals were fed and watered *ad libitum*. A 20-cm polyethylene catheter (PE 50) was inserted into the right common carotid artery under Nembutal anesthesia as previously described (6). The catheters were passed subcutaneously, exteriorized and coiled immediately posterior to the shoulders. Catheters were filled with heparinized saline (200 USP units/ml) and sealed with heat. Patency was maintained by daily flushing with 200 USP units of heparin. Blood was drawn through a 23-gauge needle inserted into the opened catheter 48 hr after surgery. Mean arterial blood pressure was recorded from the same cannula with a

physiograph pressure transducer and recorder.

**Experimental protocol.** A baseline sample of 400  $\mu$ l of blood for measurement of thyroxine, LH, TSH and PRL was withdrawn from 14 SHR and 14 control rats 60 min after the animals' cannulas were opened. During the sampling, they were allowed to move about fully in their cages and appeared calm. In all experiments the intravascular volume was maintained by replacement with normal saline. Six of the SHR and 6 controls received TRH (10  $\mu$ g/kg) injected and flushed through the catheter. Blood samples (400  $\mu$ l) for TSH and PRL determination were withdrawn from the catheter at 10, 15, 30, and 45 min after TRH injection.

**Assays of  $T_4$ , TSH, PRL and LH.** Measurement of  $T_4$  was performed by a radioimmunoassay technique employing dextran-charcoal to separate bound from free  $T_4$ , as previously described (7). Serum TSH was measured by a double antibody method using reagents provided by the NIAMDD. NIH Rat TSH-RP-1 was the reference preparation. Serum PRL was measured by a double antibody radioimmunoassay using reagents provided by the NIAMDD. NIH  $\gamma$  PRL-RP-1 served as the reference preparation. Serum LH was measured by a double antibody radioimmunoassay using reagents provided by the NIAMDD, with NIH Rat LH-RP-1 serving as the reference preparation. All measurements of each hormone were performed in duplicate in the same assay to avoid inter-assay variation.

Statistical differences between the responses of SHR and controls were evaluated with Student's *t* test for unpaired data.

**Results.** The mean baseline serum  $T_4$  of the SHR group ( $3.1 \pm 0.2$   $\mu$ g/dl) was similar to that of the controls ( $3.0 \pm 0.2$   $\mu$ g/dl). Figure 1 shows that the mean baseline serum TSH levels were higher ( $P < 0.05$ ) for the SHR group ( $1700 \pm 325$  ng/ml) than for the control

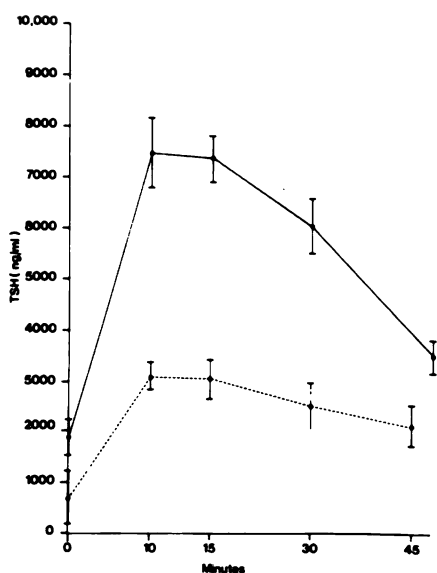


FIG. 1. Mean serum TSH responses to TRH (given at 0 time) in six spontaneously hypertensive rats (solid line) and in 6 normotensive Wistar-Kyoto rats (broken line); vertical bars show SEM.

group ( $718 \pm 3.4$  ng/ml). The maximal  $\Delta$ TSH (difference between peak responses and baseline levels) in response to TRH was greater ( $P < 0.01$ ) for the SHR ( $6362 \pm 549$  ng/ml) than for the controls ( $2760 \pm 549$  ng/ml).

Figure 2 shows that the mean baseline serum PRL levels were higher ( $P < 0.05$ ) for the SHR ( $26.1 \pm 2.1$  ng/ml) than for the controls ( $16.3 \pm 2.8$  ng/ml). The  $\Delta$ PRL response to TRH was greater ( $P < 0.025$ ) for the SHR ( $12.0 \pm 0.8$  ng/ml) than for the controls ( $6.2 \pm 1.9$  ng/ml).

Figure 3 shows that the mean baseline serum LH for the SHR ( $45.6 \pm 12.5$  ng/ml) was not significantly different from that of the controls ( $41.5 \pm 12.4$  ng/ml). The  $\Delta$ LH in response to LHRH was less ( $P < 0.001$ ) for the SHR ( $126 \pm 12.8$  ng/ml) than for the controls ( $252 \pm 24.8$ ).

The mean arterial blood pressure for the SHR group ( $159 \pm 8.6$  mm Hg) was greater ( $P < 0.05$ ) than for the Wistar control group ( $110 \pm 6.1$  mm Hg).

**Discussion.** The results of this study suggest that spontaneously hypertensive rats (SHR) display elevated basal serum levels of TSH and PRL exaggerated TSH and PRL responses to TRH. These data cannot be ex-

plained by differences in thyroid status since the serum  $T_4$  levels were similar to the SHR and control group, a finding in contrast with previous studies which reported significantly lower  $T_4$  levels in the SHR (8, 9).

Although the baseline serum LH levels were not significantly different in SHR, the LH response to LHRH was significantly less

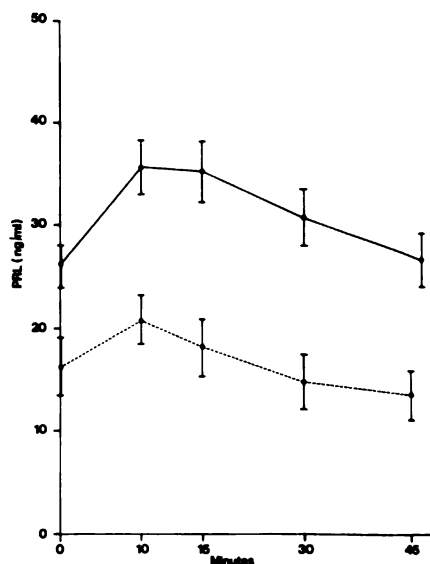


FIG. 2. Mean serum PRL responses to TRH (given at 0 time) in six spontaneously hypertensive rats (solid line) and in six normotensive Wistar-Kyoto rats (broken line); vertical bars show SEM.

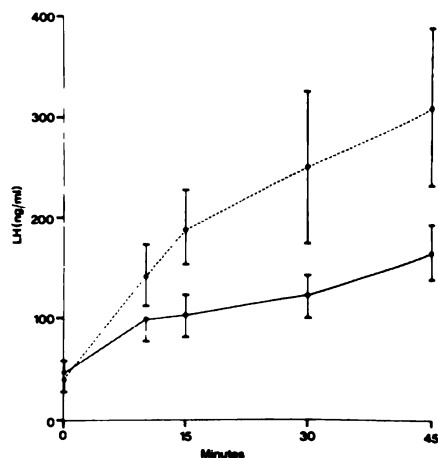


FIG. 3. Mean serum LH responses to LHRH (given at 0 time) in eight spontaneously hypertensive rats (solid line) and in 8 normotensive Wistar-Kyoto rats (broken line); vertical bars show SEM.

in the SHR than the controls. The composite findings of greater pituitary basal and stimulated TSH and PRL release and suppressed LH response to LHRH is consistent with altered central dopamine metabolism. Dopamine inhibits PRL and TSH release from the pituitary and has been reported to both stimulate and inhibit LH release under different experimental conditions (10-13). Thus, altered synthesis or turnover of dopamine in the hypothalamus of SHR could account for these observations. Although decreased levels of noradrenaline have been found in the hypothalamus of young SHR (14), there are no reports of hypothalamic dopamine levels nor dopamine turnover studies in the SHR.

Results of previous studies suggest that central dopaminergic activity may be involved in blood pressure regulation (15, 16). That the central dopaminergic system plays a direct role in blood pressure regulation is suggested by animal studies showing that the antihypertensive effect of L-dopa is associated with an accumulation of catecholamines in the cerebral parenchyma (15) and a decrease in central sympathetic outflow (16). It is thus possible that altered central dopaminergic activity in the SHR could contribute to the development of hypertension as well as the alterations in pituitary release of TSH, PRL and LH observed in this study.

**Summary.** The LH response to LHRH and the TSH and PRL response to TRH were examined in spontaneously hypertensive rats and normotensive control Wistar rats to determine if the pituitary response to these releasing hormones is altered in the hypertensive rats. Although basal levels of LH were similar in the two groups of rats, the LH response to LHRH was significantly less in the hypertensive rats than in the normotensive controls. The spontaneously hypertensive rats had higher basal levels of TSH and

PRL and significantly greater TSH and PRL responses to TRH. The results of this study suggest that the hypothalamo-pituitary axis is altered in the spontaneously hypertensive rat.

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## Epidermal Growth Factor Stimulates Ornithine Decarboxylase Activity in the Digestive Tract of Mouse (40357)

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Urogastrone (UG), extracted from human urine, and epidermal growth factor (EGF), extracted from mouse salivary glands, are polypeptides that have the same biologic actions and are highly homologous in amino acid sequence (1). Both molecules have 53 amino acid residues of which 37 are identical. It is reasonable to assume that the differences in amino acid sequence between UG and EGF are species differences and that within any one species urinary UG and salivary EGF will probably be found to be identical.

In 1938, Sandweiss and colleagues, noting that pregnant women have a low incidence of duodenal ulcer disease, demonstrated that extracts from the urine of pregnant women promoted the healing of experimentally produced (Mann-Williamson) ulcers in dog (2). Soon afterwards, urine extracts from normal men and women as well as from pregnant women were shown to contain a potent inhibitor of gastric acid secretion to which the name urogastrone was given (uro-urine, gastr-stomach, one-inhibitor) (3).

In 1975, H. Gregory reported the amino acid sequence of purified urogastrone (1). He recognized that urogastrone was highly homologous with another polypeptide, epidermal growth factor, described by Savage and Cohen in 1972 (4).

Epidermal growth factor stimulates proliferation and keratinization of epidermal tissue and promotes precocious eye opening and tooth eruption in neonatal mice. In addition, EGF has been shown to stimulate epithelial cell proliferation in cultured chick, mouse and human cells (5). Finally, EGF has been shown to increase L-ornithine carboxylase (EC 4.1.1.17) activity in mouse skin (6). This enzyme, ornithine decarboxylase, is an important step in the biosynthetic pathway of the polyamines—putrescine, spermidine and spermine (7). Polyamine production is an index of tissue growth since induction of these substances is closely related to the burst of

intracellular activity preceding actual cell synthesis.

Mouse salivary gland EGF and human urinary UG share all of the biologic actions for which they have been tested. Thus, mEGF inhibits gastric acid secretion as effectively as hUG in rats and dogs. Conversely, hUG is equipotent with mEGF in causing precocious eye opening in newborn mice and in stimulating uptake of an amino acid and in displacing labeled UG or EGF from receptor sites in cultured human fibroblasts (8).

Since UG has certain gastrointestinal actions such as inhibition of gastric acid secretion and stimulation of healing of experimental ulcers, it seems reasonable to inquire whether UG and EGF stimulate epithelial growth of the gastrointestinal tract as they do in the epidermal structures.

To examine this question, Stastny and Cohen's model of induction of ornithine decarboxylase by mouse EGF in neonatal mice was employed (6).

**Materials and methods.** Eight day old mice paired by weight from the same litter were injected subcutaneously on the dorsal surface using a 27 gauge needle with either mEGF ( $6 \mu\text{g g}^{-1}$  body wt in water given as a solution containing  $220 \mu\text{g ml}^{-1}$ ) or an equivalent volume of water for the control animals. The EGF used was generously provided by H. Gregory, ICI Pharmaceuticals, England. The mice were then returned to their mother where apparent normal feeding patterns continued.

Four hours later the animals were killed by cervical compression and 10–20 mg tissue samples were removed for study from the stomach (whole organ), duodenum (pylorus to 2 cm distal), midgut (from 7 to 10 cm distal to pylorus), colon (mid-cecum to rectum) and heart. The samples were homogenized in all glass tissue grinders (Ten Boeck type) in 50 mM sodium–potassium phosphate buffer ( $9 \text{ vol g}^{-1}$ ), pH 7.2, containing 1 mM EDTA

enediamine-tetraacetic acid-disodium and 5 mM dithiothreitol, then centrifuged at 100,000g for 15 min. Samples from supernatant were added to incubation containing 0.2 mM pyridoxal-5-phosphate, 0.5 mM L-ornithine and 0.5  $\mu$ Ci DL 1- $^{14}$ C-ornithine in a total volume of 0.5 ml of incubation buffer. "Blanks" were without tissue extract or with heat inactivated tissue extract. Upon release of  $^{14}$ CO<sub>2</sub>, a plastic cup containing 10 ml piece of cotton impregnated with 0.2 M NaOH (New England Nuclear), along with alkaline tissue solubilizer was supplied above the incubate by a glass nail. The system had an air tight seal and was incubated at 37°. To insure complete CO<sub>2</sub> release, the incubation mixture was acidified with 0.5 ml of 0.5 N HClO<sub>4</sub> for 60 min. Samples were then transferred to liquid scintillation vials and counted. CPM's were converted to equivalent quantities of CO<sub>2</sub> and expressed as pmoles of CO<sub>2</sub> liberated from 1- $^{14}$ C-ornithine per mg protein or tissue wet weight per hour incubation. Student's paired *t*-test was used for statistical analysis.

**Validation.** Figure 1 demonstrates the relationship between quantity of various tissues studied and enzyme activity and shows a linear relationship between the duration of incubation and enzyme activity. The colon demonstrated a non-linear activity increase after 20 min incubation time. L-methyl-ornithine, a competitive inhibitor of L-ornithine decarboxylase was used to establish the specificity of the enzyme from various tissues (9). To produce 50% inhibition

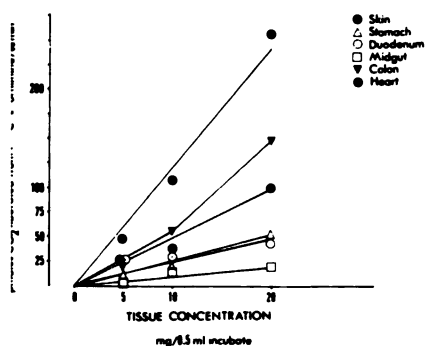


Fig. 1. Relation between enzyme activity (pmoles CO<sub>2</sub> liberated from 1- $^{14}$ C-L-ornithine per 60-min incubation) and concentrations of tissue homogenates from stomach, duodenum, midgut, colon, heart and skin.

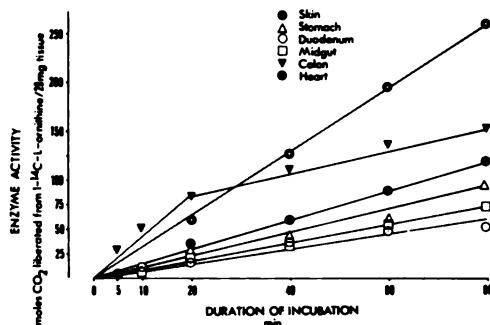


Fig. 2. Relation between enzyme activity (pmoles CO<sub>2</sub> liberated from 1- $^{14}$ C-L-ornithine per 20 mg tissue samples) and duration of incubation for the same tissues as in Fig. 1.

under our incubation conditions, the  $\alpha$ -methyl-ornithine concentrations required were: stomach,  $4 \times 10^{-3}$  M; duodenum and midgut,  $1.8 \times 10^{-3}$  M; heart,  $1.5 \times 10^{-3}$  M; and colon,  $4 \times 10^{-3}$  M.

Initial experiments using homogenates of ventral surface skin demonstrated a significant rise in ornithine decarboxylase ( $13.0 \pm .61$  nmoles CO<sub>2</sub> liberated from 1- $^{14}$ C-L-ornithine per mg protein in the EGF group versus  $9.2 \pm .56$  in the control group;  $N = 10$ ,  $P < .01$ ), confirming the results of Stastny and Cohen.

**mEGF experiment.** Results are shown in Fig. 3. In the animals pretreated with mEGF there was a significant elevation of ornithine decarboxylase activity in two tissues, the stomach and the duodenum. The increases in the midgut and the colon were not statistically significant. The control tissue, heart, demonstrated no difference.

**Discussion.** From these results it is concluded that EGF, and therefore probably also UEG, stimulates an increase in ornithine decarboxylase activity in the stomach and duodenum of neonatal mice. This suggests a possible physiologic role for EGF in controlling mucosal growth in the proximal digestive tract.

It is of interest that in the control tissue, heart, ornithine decarboxylase can be induced by another stimulus, stress, in the form of aortic constriction (10).

A further hypothesis is suggested from this study. Human urogastrone has been identified by immunofluorescent techniques in the salivary glands and duodenal Brunner's

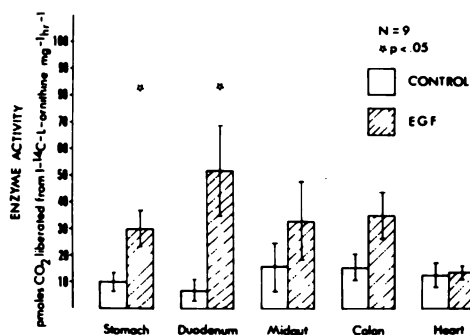


FIG. 3. Enzyme activity (pmoles CO<sub>2</sub> liberated from 1-[<sup>14</sup>C]L-ornithine per mg wet weight tissue per hour incubation) 4 hr after subcutaneous administration of mEGF (6 μg g<sup>-1</sup> body wt) or equivalent volume of water.

glands of man (11). This latter location is the most common site for peptic ulceration. Since an increase in secretion of acid and pepsin is not present in many ulcer patients, a decrease in a hypothetical "tissue resistance factor" is assumed to be involved. The nature of this factor is not clear but this study suggests that urogastrone should be considered as a candidate for this role.

**Summary.** This study examined the effect of EGF (6 μg g<sup>-1</sup> body wt, subcutaneously) on OD concentration in stomach, duodenum, midgut and colon, as well as a control tissue, heart, in 8-day-old mice. The animals were killed 4 hr after either EGF or control water injections. OD activity, expressed as picomoles of <sup>14</sup>CO<sub>2</sub> liberated from 1-[<sup>14</sup>C]L-ornithine per mg wet weight tissue, was significantly higher in the animals given EGF than in controls in the stomach (EGF 29.9 ± 6.8; control 9.9 ± 3.6, *P* < .05) and the duodenum

(EGF 51.7 ± 16.9; control 6.5 ± 4.3, *P* < .05) but not in the midgut, colon or heart. It is concluded that epidermal growth factor stimulated ornithine decarboxylase activity in the stomach and duodenum of neonatal mice suggesting a possible role for EGF (or urogastrone) in mucosal repair and defense in these tissues.

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Inhibition of  $\beta$ -Glucuronidase Activity by Albumin of Human Synovial Fluid (40358)L. MORO,<sup>1</sup> B. DE BERNARD, P. INAUDI, AND F. GONANO*Istituto Regionale Medicina Fisica e Riabilitazione, Laboratorio di Patologia Clinica, Udine (Italy)*

The course of a study on the kinetic properties of  $\beta$ -glucuronidase (EC 3.2.31) of synovial fluid evidence was obtained showing the presence of an endogenous inhibitor of this enzyme (1). The interest for this finding is enhanced by the fact that cartilage erosion in inflammatory joint diseases is considered to be caused by the degradative action of various enzymes on the constitutive connective tissue (2, 3). According to these views the extent of this digestion also depends on the level of specific inhibitors of the various degradative enzymes. An inhibitor of chondromucoproteinase (4). An inhibitor of collagenase, which is present in synovial fluids of rheumatoid arthritic patients (5, 6), has been found both in synovial fluid and serum (7). Furthermore, two inhibitors of proteinases, which are biologically identical to serum  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT) and  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M), have been detected in human synovial fluid.

The presence of an inhibitor of  $\beta$ -glucuronidase, an enzyme which participates in the metabolism of glycosaminoglycans in a connection with hyaluronidase (9), suggests that a large spectrum of enzyme activities are present in the extracellular compartments of connective tissue. This report describes a preliminary study for the purification of the inhibitor from synovial fluid and the analyses carried out to identify the compound.

**Experimental procedures.** *Synovial fluid collection.* Human synovial fluids were obtained from the knee joint of patients with inflammatory and degenerative disorders under aseptical conditions and frozen at  $-20^\circ\text{C}$ . The samples were thawed, freed of cells and elements by centrifugation, and dialyzed with bacterial hyaluronidase (Miles-

Servac, USA) as previously reported (1). After dialysis, synovial fluids were fractionated by gel filtration through a column of Sephadex G-200 ( $90 \times 2.5$  cm). Proteins were eluted with 20 mM Tris-HCl buffer, pH 8, containing 0.17 M NaCl and 10 mM  $\text{CaCl}_2$ , at a flow rate of 6 ml/hr. Fractions of two ml were collected, pooled (as indicated in the Results section), concentrated by ultrafiltration and examined for inhibitory activity. Further purification of the proteins with the lowest molecular weight was achieved by ion exchange chromatography in a column of DEAE A 52 ( $14 \times 2$  cm), equilibrated with 50 mM Tris-HCl buffer, pH 8. Elution with this buffer was followed by a NaCl gradient elution, at a flow rate of 24 ml/hr. Fractions of two ml were collected, pooled (as indicated in the Results sections), concentrated by ultrafiltration and tested for their inhibitory capacity of  $\beta$ -glucuronidase activity.

**Enzyme assay.** The inhibition of the  $\beta$ -glucuronidase activity by the synovial fluid and by the fractions isolated therefrom was routinely assayed by using the Helix pomatia enzyme (glucuronidase, ENDO, USA). The following commercial human serum albumins have been used in the studies of the inhibition of the enzyme: Human Albumin (95-100%), from Immununo-Oesterreiches Institut fuer Haemoderivative Ges.; Human Albumin (fatty acids free) from fraction V (SIGMA, USA). Occasionally, the extent of inhibition was also tested on a partially purified endogenous  $\beta$ -glucuronidase. The enzyme assay (0.2 ml) was carried out with phenolphthalein- $\beta$ -D-glucuronide as substrate (1).

**Analytical procedures.** Dialysis was performed first against the buffer solutions and then exhaustively against deionized water. Ultrafiltration was performed using Amicon PM 30 membranes.

$\alpha_2$ -M and  $\alpha_1$ -AT were quantitatively evaluated by single radial immunodiffusion using immunokits from Behringwerke. Proteins were determined by the method of Lowry *et*

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al. (10), by using bovine serum albumin (BSA) as standard.

Electrophoresis on cellulose acetate strips was carried out at 1.5 mA/cm for 20 min in Tris-Barbital buffer (Gelman Instrument Co., MI) pH 8.8 ( $\mu = 0.06$ ). Staining was performed by soaking the strips in a 5% TCA solution containing 0.5% of Ponceau-S stain (Gelman) for 20 min. Destaining was performed by soaking the strips in 5% TCA.

Electrophoresis for the immunoassays (2% agar) were performed in Tris-Barbital buffer pH 8.4 ( $\mu = 0.06$ ) for 50 minutes at 50 V and 6 mA. Rabbit total antiserum (50  $\mu$ l) was incubated in the troughs at room temperature for 18 hr. At the end of the electrophoresis, the plates were washed for 8 hr with several changes of physiological solution and then dried over a blotting paper under a gentle stream of air for 2 hr. Staining was performed in methanol/5% acetic acid (10:90, v/v) containing azo-carmin G (Geigy). Destaining was accomplished by soaking the plates in 5% acetic acid.

Preparative polyacrylamide gel electrophoresis was carried out according to Sottocasa *et al.* (11).

**Results.** Gel filtration through Sephadex

G-200 of human synovial fluids digested with hyaluronidase resulted in the separation of three peaks.  $\alpha_2$ -M, synovial fluid  $\beta$ -glucuronidase and  $\alpha_1$ -AT were recovered in peaks I, II, III respectively (Fig. 1). Inhibition of snail juice  $\beta$ -glucuronidase activity was exhibited only by the pooled fractions of peak III.

After concentration by ultrafiltration and extensive dialysis these fractions were applied to a column of DEAE A 52. The elution profile of this column is shown in Fig. 2. The small amount of protein eluted with Tris buffer did not show any inhibitory activity. The NaCl gradient separated a single peak, which contained the  $\beta$ -glucuronidase inhibitor and was devoid of any  $\alpha_1$ -AT activity.

When compared to the synovial fluid and peak III of the gel filtration, the peak eluted from the DEAE column with the NaCl gradient (DEAE peak) exhibited a two-fold and four-fold increased inhibitory activity, respectively (Table I). It also showed a marked inhibition of the endogenous  $\beta$ -glucuronidase present in peak II of the gel filtration.

The DEAE peak was analyzed by electrophoresis on cellulose acetate. The electropherogram, stained for proteins, is shown in Fig. 3. A single protein band was observed.

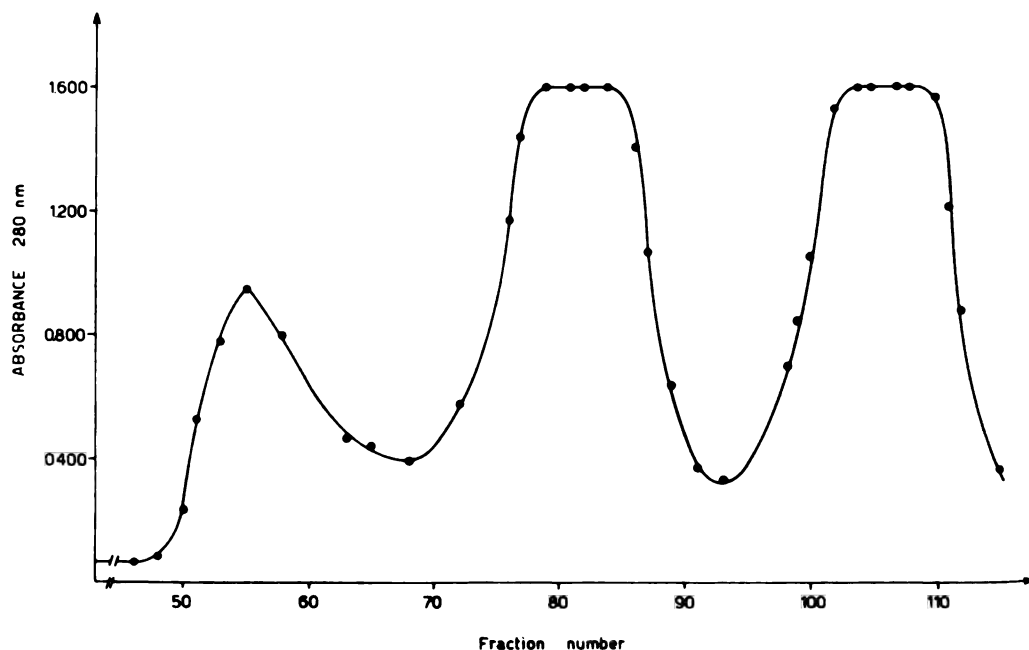


FIG. 1. Gel filtration of human synovial fluid on Sephadex G-200. Peak I = fractions 50-60; peak II = fractions 75-90; peak III = fractions 100-105. (For details, see Experimental Procedures).

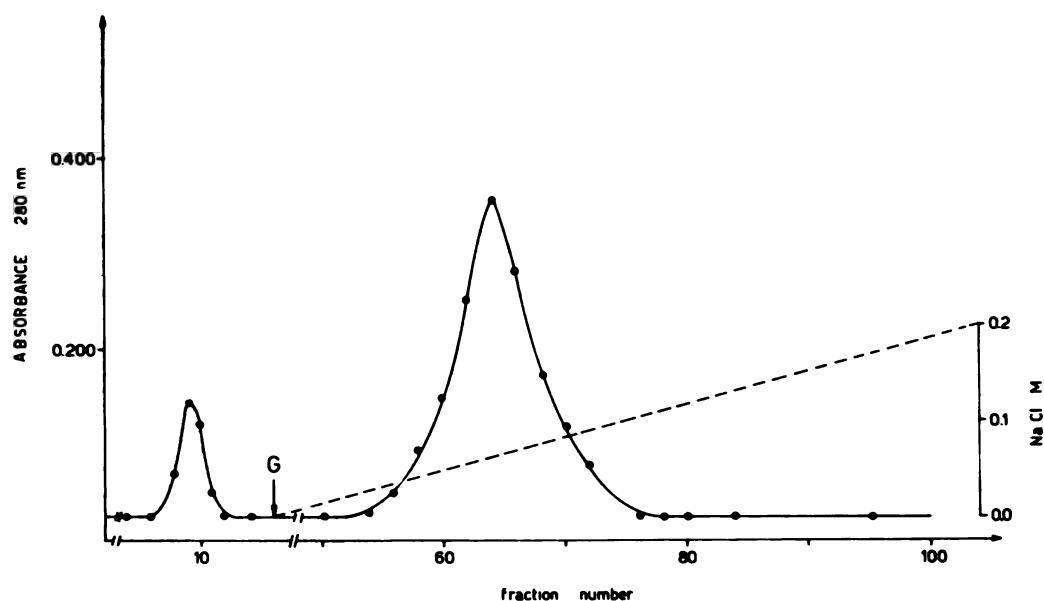


FIG. 2. Separation of the  $\beta$ -glucuronidase inhibitor by ion exchange chromatography on DEAE A 52. (For details, see Experimental Procedures).

TABLE I. INHIBITION OF  $\beta$ -GLUCURONIDASE BY PROTEIN FRACTIONS DERIVED FROM HUMAN SYNOVIAL FLUID.

Purification step	mg of protein/assay giving 50% inhibition
Synovial fluid	2.3
Peak III of Sephadex G-200	1.1
DEAE A 52 (NaCl gradient elution)	0.56

which migrated to a position corresponding to albumin of a serum sample analyzed in a parallel run.

By immunoelectrophoresis, the DEAE peak reacted as serum albumin (Fig. 4), giving a single symmetrical precipitin arc with rabbit antiserum to human serum.

By preparative polyacrylamide gel electrophoresis, the DEAE peak provided five subfractions (Fig. 5). Each subfraction inhibited the  $\beta$ -glucuronidase activity and reacted as serum albumin when tested by immunoelectrophoresis. In order to further demonstrate that albumin is the true inhibitor, we have tested also two commercial purified preparations of the compound as illustrated by Fig. 6. From the figure it appears that both preparations inhibit  $\beta$ -glucuronidase activity.

**Discussion.** Previous studies (1) have shown that the synovial fluid contains an inhibitor of  $\beta$ -glucuronidase, which exerts a competitive type of inhibition on the activity of both snail juice and rat liver enzyme. This inhibitor has now been purified and shown to be the albumin present in synovial fluid. The identification of albumin as the inhibitory substance is based on a comparison between the purified inhibitor and human serum albumin carried out by electrophoretic and immunologic techniques.

Albumin is known for its capacity of binding a number of small molecules. Hence, the inhibition of  $\beta$ -glucuronidase could be due to one such molecule and not to the protein itself. This possibility seems, however, unlikely since we have previously shown that a protease treatment of synovial fluid completely abolishes the inhibitory activity (1). Furthermore the results obtained by subjecting the inhibitor to the polyacrylamide gel electrophoresis indicate that the protein dissociate into five subfractions: each one, however, reacts with antibody to human serum albumin and inhibits  $\beta$ -glucuronidase. The fact that human albumin may be heterogeneous in purified preparations and in the serum itself has been already reported in literature (12). This microheterogeneity of hu-

man serum albumin may be directly transferred to the albumin of human synovial

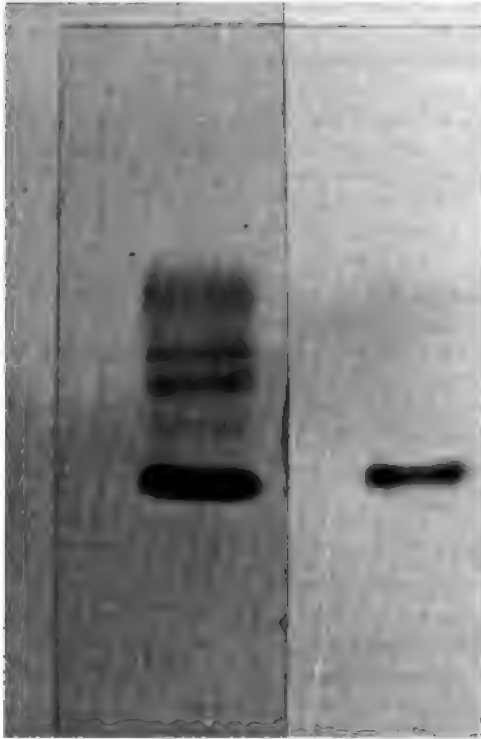


FIG. 3. Electrophoresis on cellulose acetate of serum proteins (left) and of combined fractions of the peak eluted from DEAE column (right).

fluid, since plasma proteins reach the synovial space by diffusion (13). It appears therefore that inhibition of  $\beta$ -glucuronidase activity is shown also by the purest fractions of human albumin as those obtained by gel electrophoresis.

Preparations of  $\beta$ -glucuronidase of high specific activity are stabilized in the assay by additions of 0.01% bovine serum albumin (14). This protective effect of albumin is apparently in contrast with our finding. One has, however, to consider that albumin of synovial fluid exhibits a competitive inhibition, which might not be seen in the usual assay conditions. However, in our experimental conditions, also commercial preparations of human serum albumin have been shown to be inhibitors of the enzyme activity (Fig. 6). This fact is of special interest since it has been reported (15) that commercial serum albumin preparations, usually stored for various periods of time by the manufacturing supply houses, may undergo alterations during storage, which might affect the biological properties of albumin in metabolic studies.

The human blood serum contains a number of high-molecular weight components, which inhibit hydrolytic enzymes such as collagenase, proteinases and other degrading enzymes (16–18). The demonstration that albumin can inhibit synovial fluid  $\beta$ -glucuron-

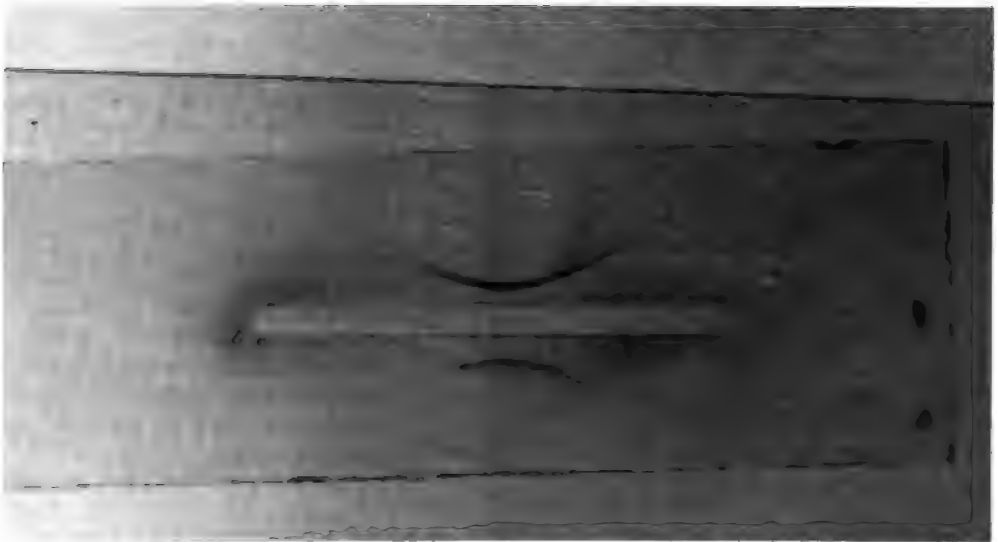


FIG. 4. Immunoelectrophoresis of combined fractions of the peak eluted from DEAE column (upper precipitation arc) and of serum albumin (lower arc). Antibody trough contained antiserum to whole serum proteins.

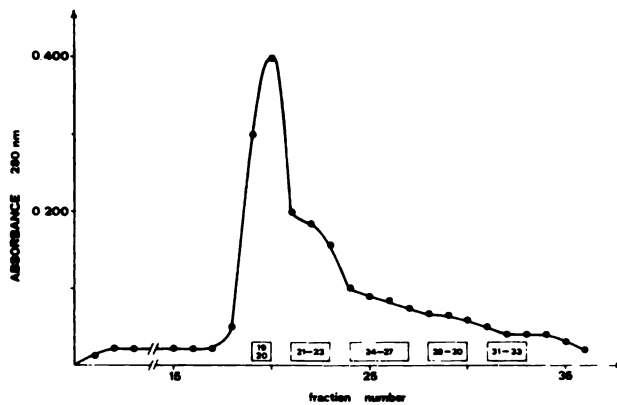


FIG. 5. Preparative polyacrylamide gel electrophoresis of combined fractions of the peak eluted from DEAE column.

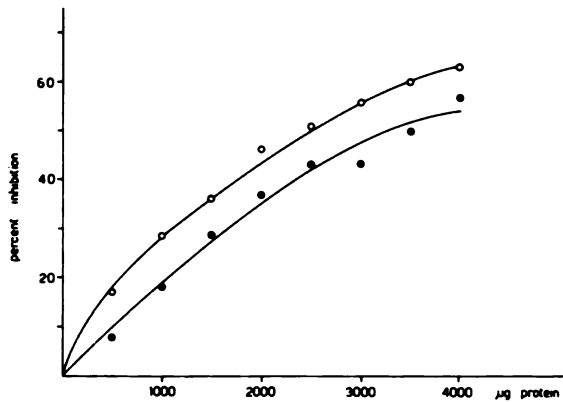


FIG. 6. Inhibition of snail juice  $\beta$ -glucuronidase by: O = human albumin from SIGMA; ● = human albumin from Immuno Oesterreiches Institut.

idase suggests that the serum proteins released into the inflammatory fluid can modulate a wide spectrum of degenerative reactions.

**Summary.** From human synovial fluid a protein inhibiting  $\beta$ -glucuronidase activity has been extracted and purified. The inhibitor is shown to be the albumin present in the synovial fluid. The identification of albumin is based upon a comparison between the purified inhibitor and human serum albumin carried out by electrophoretic and immunologic techniques.

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## Pituitary Cell Transplants to the Cerebral Ventricles Promote Growth of Hypophysectomized Rats<sup>1</sup> (40359)

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Surgical removal of the adenohypophysis in animals results in retarded growth as well as decline in peripheral endocrine function. Attempts at demonstrating recovery of growth by means of heterotopic pituitary transplants have met with only limited success (1-5). For example, Halasz and associates reported that transplantation of a whole pituitary gland into the hypophyseal area of the brain resulted in partial restoration of growth (3). Growth restoration of a smaller magnitude was observed after transplantation of pituitary glands to remote sites as the renal capsule (2) or into the chamber of the eye (1), as well as intramuscular (5) or subcutaneous (4) placement. Gittes and Kastin (5) observed a logarithmic relationship between growth and number of intramuscular glands, and by extrapolation concluded that 750 glands would be needed for restoration to normal growth. Kragt and Kragt (4), on the other hand, observed partial restoration of growth (46%) in young (37-day-old) hypophysectomized male and female rats bearing a single subcutaneous pituitary gland for 30 days.

The ease with which pituitary glands can be automatically dispersed to yield suspensions of single viable cells is now widely appreciated. In addition to their usefulness in *vitro* studies, these single cell suspensions have also been implanted into the kidney capsule or hypophysiotropic area of hypophysectomized rats (6, 7). On the basis of morphological data it was suggested that such transplanted cells retained functionality *in vivo*.

There is increasing evidence that cerebrospinal fluid (CSF) contains (hypothalamic) neurohormones (8) which may participate in the regulation of pituitary function (9). In the present study, the ventricular system of the brain of hypox rats was therefore chosen as the implantation site for dispersed pituitary cells, and the restoration of body growth was used as an index of functionality of the implanted cells.

**Materials and methods.** In the usual experiment, hypox Sprague Dawley male rats weighing 80-100 g (~30 days old) were purchased from Charles River Breeding Laboratories, Inc., (CD Strain (Outbred Albino), Wilmington, MA) and permitted one week of postoperative recovery. In some cases, sham-hypox littermates were also used. Twenty-gauge hypodermic needles, filed to an unbevelled end 3.25 mm in length and filled with silastic, were stereotactically implanted into the left ventricle and anchored with acrylic cement according to the procedures of Severs *et al.* (10). Animals were maintained one additional week prior to cell implantation. During this period, animals showing increases in body weight of >5% over initial postoperative levels, suggestive of incomplete hypophysectomy, were discarded from the experiment. Anterior pituitaries from donor males of the same strain (CD, 250-400 g, >70 days) were dispersed in trypsin (11), counted, and resuspended in "mock CSF", consisting of 16 mg dextrose, 176 mg NaHCO<sub>3</sub>, 15 mg KCl, 14.0 mg CaCl<sub>2</sub> (anhydrous), 8.1 mg NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 23.5 mg MgCl<sub>2</sub>·6H<sub>2</sub>O, 13 mg urea, 91 mg NaCl in 100 ml double-distilled water. Each animal received a single injection of 10-20  $\mu$ l either "mock CSF" (control) or 1-3  $\times$  10<sup>6</sup> cells prepared in CSF vehicle (experimental), delivered via the needle of a microliter syringe

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through the silastic plug of the indwelling cannula. The quantity of cells delivered was equivalent to approximately  $\frac{1}{4}$ – $\frac{3}{4}$  of a whole pituitary gland. Three to 6 animals were used per group. The animals were maintained with 5% glucose in their drinking water and allowed lab chow *ad libitum*, under a 12-hr light (0600–1800) cycle, for periods up to 3 months. They were weighed 3 times per week.

In one experimental series, body composition analysis was done according to the procedure of Hartsook and Hershberger (12). The experimental protocol involved analysis of 12 hypox rats (80–120 g) 2 weeks postsurgery (group A) and 12 hypox littermates which had received either "CSF" or  $2 \times 10^6$  cells 2 weeks postsurgery followed by a 30-day growth period (group B). Regression analysis of the body composition data from group A gave the following equations for prediction of initial body compositions of animals in group B: Dry matter =  $0.34 (BW) - 4.77$  [ $r^2 = .95$ ]; Lipid =  $0.1 (BW) - 4.07$  [ $r^2 = .79$ ]; Ash =  $.04 (BW) - .31$  [ $r^2 = .90$ ]; Protein =  $0.2 (BW) - .71$  [ $r^2 = .94$ ]. This protocol permitted evaluation of changes in body composition over the growth period.

Growth hormone (GH) was measured with a double antibody radioimmunoassay procedure (sensitivity, 3 ng/ml) using materials provided by the NIAMDD (Rat Pituitary Program). Protein content of brain homoge-

nates was estimated by the Lowry procedure (13).

Growth curves were analyzed by the variance ratio test on double reciprocal plots of log weight gain vs. log time. This transformation yielded linear graphs and randomly scattered residual variance plots. Comparative growth responses at 30 days postimplantation, as well as other data (bone lengths, body composition and hormone levels) were analyzed by ANOVA or, when appropriate, Student's *t* test.

**Results. Growth response.** During the first 3-week postimplantation period, growth of hypox animals bearing  $1 \times 10^6$  cells, expressed as % weight gain, was similar to that of sham-hypophysectomized littermates (Fig. 1). After this time growth tended to plateau (see Fig. 1, Exp. #1 and #2,  $1 \times 10^6$  cells). The growth response was related to the number of cells implanted. At no time did total growth exceed that of the animal with an intact pituitary; however, animals receiving more cells tended to plateau later. A single injection of  $3 \times 10^6$  cells resulted in a doubling of the animals' body weight over a period of 3 months (Fig. 1, insert). Implantation of  $1 \times 10^6$  cells into the ventricles of *nonhypophysectomized* rats resulted in slightly but significantly ( $P < .05$ ) depressed growth curves.

There was an increase in tibial and femoral

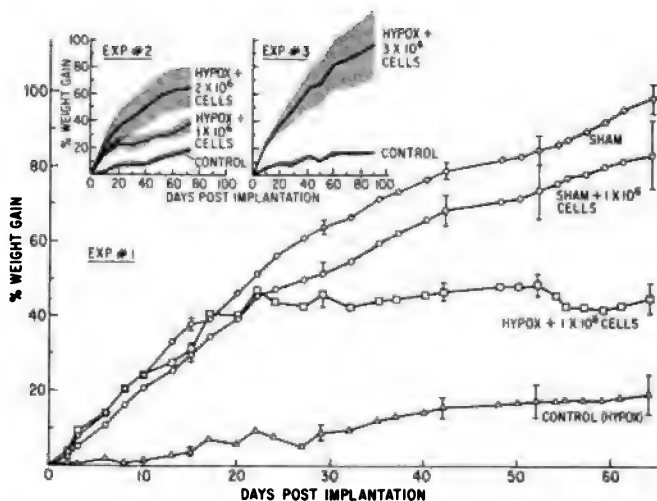


FIG. 1. Exp. #1, percent increase in body weight of ~30-day male hypox rats receiving a 10  $\mu$ l intraventricular injection of either "mock" cerebral spinal fluid (CSF control) or  $1 \times 10^6$  single pituitary cells from 70-day-old donors (bottom two lines) or sham hypophysectomized littermates  $\pm 1 \times 10^6$  pituitary cells (top two lines). Each line corresponds to the weight gain of 4 animals; error bars and shading represent  $\pm$  SEM. Effect of implantation of  $1 \times 10^6$  (Exp. #2) or  $3 \times 10^6$  cells (Exp. #3) on weight gain is shown in the inserts.

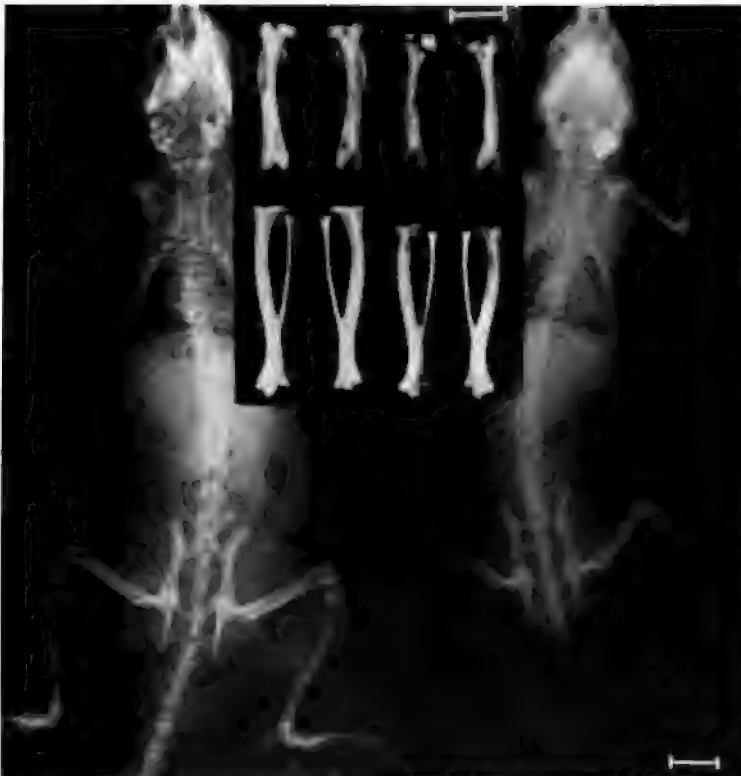
lengths measured either radiographically or on bones dissected from the rats at autopsy (see Fig. 2). In both cases bones were significantly ( $P < .05$ ) longer in the experimental group. There was a positive correlation between the two methods of measurement. Actual tibial, femoral, and pelvic lengths were  $31.5 \pm (\text{SEM}) .29$ ,  $26.0 \pm .33$ ,  $\pm .24$  mm respectively for controls and  $\pm .88$ ,  $28.9 \pm .24$ ,  $33.3 \pm .44$  mm for experimentals ( $1 \times 10^6$  cells). Correlations of x-rays were  $r = .77$  (tibia),  $r = .84$  (femur),  $r = .97$  (pelvis).

**Body composition.** In a separate experiment, 30-day-old hypox  $\delta$  rats receiving  $2 \times 10^6$  cells intraventricularly showed weight over 30 days of  $22.9 \pm 0.5$  g (SEM) (6% increase in body weight) vs.  $7.5 \pm 1.4$  g (1% increase in body weight) for those receiving "CSF". The increase in the experimental group represented  $14.1 \pm 3.5$  g dry weight of which  $5.0 \pm 1.5$  g were protein,  $8.5$  g were lipid, and  $1.1 \pm 0.5$  g were ash. Increase in the control group represented

$2.8 \pm 0.5$  g dry matter of which  $0.1 \pm 0.3$  g were protein,  $2.6 \pm 0.7$  g were lipid and  $0.4 \pm 0.1$  g were ash. These results clearly show that significant ( $P < .05$ ) increases in both protein and fat account for the weight gain in the experimental animals.

**Age and sex.** Younger recipients showed a better growth response than the older ones (Fig. 3, top). Pituitary cells from older donor animals gave better responses than cells from young animals (Fig. 3, middle). Cells from male donors of different ages gave consistently inferior responses when implanted into young hypox females (Fig. 3, middle vs. bottom). This result is consistent with the observation that male rats grow larger than females. Cells from  $>70$ -day-old female donors were as effective as their male counterparts when transplanted into hypox males (data not shown).

**Somatotroph implantation.** Intraventricular implantation of 630,000 somatotrophs purified to 90% by the method of Snyder *et al.* (14) resulted in a weight gain at 30 days of



2. Radiographs and bones (tibia-lower, femur-upper) from two hypox animals 30 days after intraventricular implantation of either  $2 \times 10^6$  cells (left) or "mock" CSF vehicle (right). Scale bar equals 1 cm.



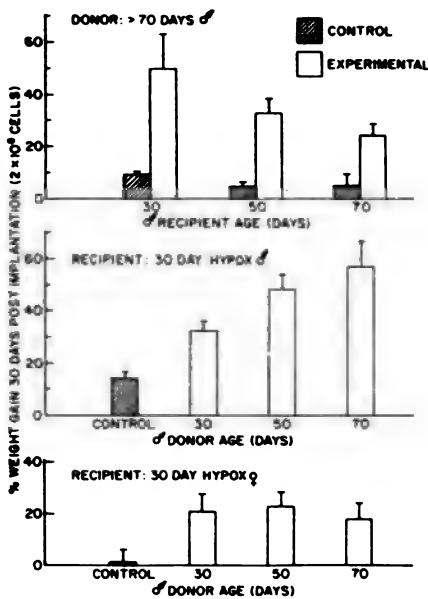


FIG. 3. Effect of age of recipient at hypophysectomy (top), and age of donor pituitary cells in ♂ recipients (middle) or ♀ recipients (bottom) on weight gain. Statistical analysis: top panel: one animal in the 30-day experimental group grew 3× more than the other three. Analysis of variance (ANOVA) on these data excluding this single animal resulted in significant ( $P < 0.05$ ) elevations in the experimental groups in all cases. Middle panel: by ANOVA 50 and 70-day old donor cells caused significant ( $P < 0.05$ ) growth. Bottom panel: growth, although apparently elevated, was not statistically significant.

17.4 ± 3.6% vs. -1.5 ± 4.3% for those injected with vehicle ( $P < .05$ ).

**Castration.** Four groups ( $n = 5$  each group) of hypox recipients, two of which were castrated at the time of pituitary removal, received either  $2 \times 10^6$  pituitary cells or vehicle. Growth (% wt. gain) 30 days postimplantation was as follows: (a) castrated animals with cells  $45.8 \pm 10.8\%$ ; (b) castrated animals with vehicle  $5.3 \pm 1.5\%$ ; (c) noncastrated animals with cells  $69.1 \pm 15.0\%$  and (d) noncastrated animals with vehicle  $9.1 \pm 2.7\%$ . Growth of animals at 30 days in both experimental groups was significantly greater than in controls ( $P < .05$ ), but not significantly different between experimental groups.

**Brain and blood growth hormone (GH).** The levels of GH in homogenates of brains prepared from animals receiving either  $1 \times 10^6$  pituitary cells or vehicle is given in Table I. The data reveal detectable hormone in the brains of the experimental group 30 days

TABLE I. GROWTH HORMONE LEVELS (ng GH/mg PROTEIN) IN BRAIN HOMOGENATES PREPARED FROM HYPOX RATS PREVIOUSLY IMPLANTED WITH  $1 \times 10^6$  PITUITARY CELLS (EXPERIMENTALS) OR CSF VEHICLE (CONTROLS).

Treatment	Days postimplantation		
	12	20	30
Experimentals <sup>a</sup>	53.4 ± 9.0 <sup>b</sup>	16.1 ± 4.1	13.6 ± 2.3
Controls	3.2 ± 3.2	0 <sup>c</sup>	0 ± 0

<sup>a</sup> 30 day old hypox ♂ rats received  $1 \times 10^6$  pituitary cells from 70-day-old ♂ rats.

<sup>b</sup> SEM.

<sup>c</sup> One animal; all other groups had three to four animals.

postimplantation, but at ¼ the level detected 12 days postimplantation.

GH levels in the sera of each of the animals in Table I were, in every case, undetectable. Possible reasons for this result are currently under study.

**Cell placement and viability.** In four separate experiments designed to assess requirements of cell placement and viability in relation to the growth response, the following data were collected (% wt. gain in 30-day-old hypox males one month postimplantation): (a)  $1 \times 10^6$  cells - intraperitoneally,  $13 \pm 4\%$ ; (b)  $1 \times 10^6$  cells - anterior chamber of the eye,  $7.8 \pm 2.4\%$ ; (c) heat-killed ( $56^\circ$ , 30 min) cells - intraventricularly,  $4.7\%$ ; and (d) a 100,000g particle fraction (prepared from  $1 \times 10^6$  cells)  $5.4 \pm 0.3\%$ . None of these responses were significantly different from vehicle-injected controls, but all were significantly lower ( $P < .01$ ) than the response obtained by implanting  $1 \times 10^6$  cells intraventricularly ( $40.6 \pm 4.0\%$ , mean of the four experiments).

**Histology.** Serial sections of the entire brains of several experimental animals revealed epithelial cells in the 3rd ventricle, lateral ventricles, and subarachnoid space. Since such cells were not found in the sections of the brains of a control animal, it is tentatively concluded that the pituitary cells spread throughout the entire ventricular system.

**Discussion.** The key finding in this study is that implantation of pituitary cells into the ventricular system of hypophysectomized rats results in animal growth. This growth is reflected both in increased bone length as well as deposition of total body protein. Our data

that intact cells placed in the ventricles required to obtain this response since cells placed in the anterior chamber, eye or peritoneal cavity nor heat-killed or pituitary organelles gave a positive response.

The growth response can probably be attributed to somatotrophs in the pituitary cell suspensions for the following reasons: first, implantation of purified somatotrophs gave a positive response; second, the response was noted in a castrated animal in which influence of anabolic steroids were not present; third, GH was detected in the brains of 30 days postimplantation of cells, but not vehicle-injected controls (Table I).

These results show that the CSF of the hypophysectomized rat provides a suitable functional milieu for maintenance of somatotrophs for at least 3 months postimplantation.

**Primary.** Implantation of acutely dissociated adenohypophyseal cells into the lateral ventricles of hypophysectomized rats resulted in partial growth restoration for periods of up to three months. Weight gain by experimental animals was consistently 20%–60% greater than among hypophysectomized controls; the response was related to the number of cells implanted. The weight gain reflected increases of both protein and fat in body composition. A significant increase in bone lengths was also observed among rats bearing intraventricular cells. Intraventricular implantation of either heat-killed antipituitary cells or subcellular organelles, or implantation of pituitary cells into the peritoneal cavity or anterior chamber of the eye did not promote significant growth in hypophysectomized recipients. The results indicate that transplanted growth hormone-

secreting cells are provided with a suitable functional milieu by the cerebrospinal fluid of the hypophysectomized rat.

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## Serologic Response of Primates to Influenza Viruses (40360)

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The appearance of a new strain of influenza A at Fort Dix, NJ, in February of 1976, was of interest, principally because of its antigenic relatedness to the virus presumed to be etiologically responsible for the 1918 pandemic, a variant of swine influenza. Very little is known regarding the interrelationships between human and animal influenza, although it has been clearly demonstrated that this virus does exist in a wide variety of animal and avian species. Only limited information, however, is available on influenza in primates other than man, and these data have generally resulted from experimental rather than natural infections (1-7), although an epidemic of influenza with high mortality was reported in baboons during the 1918-19 pandemic (WHO Ref. Z2/180/11, 16 July 1971). Other investigators (8, 9) have also reported influenza in simians, with death and clinical disease noted. Easterday (WHO Ref. Z2/180/11 and Z2/87/5, 10 January 1973) reported antibody in primates to A/FM1 at the San Diego Zoological Gardens.

Serological surveys have indicated that antibody to influenza A (PR8, FM1, Hong Kong) and influenza B (Lee) exists to varying extents in "normal" colonies of gorillas, chimpanzees, orangutans, gibbons, baboons in Africa, captive baboons, Japanese macaques, African green monkeys, marmosets, squirrel monkeys, and capuchin monkeys. Owl, howler, and spider monkeys were generally serologically negative, although the number of animals examined was very small (7).

In 1974, an outbreak of respiratory disease occurred in a group of newly imported baboons (*Papio cynocephalus*). An isolate was obtained from seven of 20 animals, which appeared to be identical to A/Mayo Clinic/4/75 (H3N2). (Dr. F. Lief, personal communication). The seven animals from which virus isolations were made had high antibody titers; of the remaining 13 animals, six had antibody titers to the virus and seven had no antibody to the isolate but developed

titers later. The serologic data suggested that infection had occurred just prior to shipment from Kenya in the late spring of 1974.

Since the data indicated the susceptibility of nonhuman primates to influenza virus following contact with infected humans, occurrence of a new strain of influenza virus offered the opportunity to examine representative simian sera in order to ascertain the possible role nonhuman primates may play in this virus infection. Reported herein are results obtained by examining human, captive-chimpanzee, and baboon sera collected each month for the year immediately following the outbreak of the A/New Jersey/76 (Hsw<sub>1</sub>N<sub>1</sub>) virus.

**Materials and methods. Sera.** Human, chimpanzee (*Pan troglodytes*), and baboon (*P. cynocephalus*) sera were obtained from randomly selected populations each month in the usual manner. Sera were so selected in order to avoid the following of animals with high titers and the possibility of not detecting seroconversions. Human donors were questioned regarding influenza vaccinations in order to distinguish vaccinees from cases (Table I). Most of the human volunteers were animal personnel or laboratory staff engaged in either the daily handling of the animals or in collecting specimens from these animals. Since the number of staff and chimpanzees is limited, over the 10-month study period of number of these were sampled on more than one occasion.

**Antigens.** Two influenza antigens supplied by CDC, Atlanta, Georgia, were used throughout the study. These consisted of chicken egg preparations of allantoic fluid and included strains A/Victoria A/3/75 (H3N2) and A/New Jersey/8/76 (Hsw<sub>1</sub>N<sub>1</sub>). Control chicken antisera to each virus, also provided by CDC, were routinely and simultaneously tested each month.

**Antibody determination.** A micro-HI test using 0.025 ml volumes and 4 HA units of antigen with 0.8% chicken erythrocytes was

Number of sera with HI titer (Cumulative numbers)

Date	Antigen	Primate Sera	<10	≥10	≥20	≥40	≥80	≥160	≥320	>320
pt. '76	Victoria	Human	0	16	14	9	3			
		Chimpanzee				NOT DONE				
		Baboon	0	10	8	5	3	1		
	New Jersey	Human	3	12	7	3				
		Chimpanzee				NOT DONE				
		Baboon	7	3						
t. '76	Victoria	Human	1	9	7	4	1	1		
		Chimpanzee	7	10	1					
		Baboon	5	38	26	10	3			
	New Jersey	Human	5	5	3	3	1			
		Chimpanzee	17							
		Baboon	42							
v. '76	Victoria	Human	2	11	6	2				
		Chimpanzee	4	9	7	2				
		Baboon	10	18	7	2				
	New Jersey	Human	4	9	9	7	6(2) <sup>a</sup>	2	2	2(1)
		Chimpanzee	8	3						
		Baboon	28							
c. '76	Victoria	Human	2	9	8	6	3	3	1	1
		Chimpanzee	11	6	2	1				
		Baboon	1	21	16	9	7	6		
	New Jersey	Human	1	10(1)	6(1)	4	2	2(2)		
		Chimpanzee	17							
		Baboon	22							
t. '77	Victoria	Human	1	9	3					
		Chimpanzee	9	8	2					
		Baboon	8	15	5	5	5	4	3	
	New Jersey	Human	6	2(1)	1(1)					
		Chimpanzee	17							
		Baboon	22	1						
b. '77	Victoria	Human	2	13	13	8	1			
		Chimpanzee	13	4	1					
		Baboon	13	29	26	21	12	5	1	
	New Jersey	Human	7	8	7	6	6	4(2)	2(2)	
		Chimpanzee	16	1						
		Baboon	42	1						
urch '77	Victoria	Human	0	17(3)	8(1)	5	5(2)	2	2	2(1)
		Chimpanzee	10	7	2					
		Baboon	8	29	21	10	4	1		
	New Jersey	Human	8	9	6(2)	4(1)	3(1)	2	2	2(2)
		Chimpanzee	17							
		Baboon	37							
ril '77	Victoria	Human	0	12	12	12	12	9	9	9(2)
		Chimpanzee	1	16	16	12	12	11	7	
		Baboon	0	25	25	23	21	21	20	20

TABLE I.—Continued.

Date	Antigen	Primate Sera	Number of sera with HI titer (Cumulative numbers)							
			<10	≥10	≥20	≥40	≥80	≥160	≥320	>320
May '77	New Jersey	Human	9	3	3	3	3(1)	2	3	2(1)
		Chimpanzee	17							
		Baboon	25							
	Victoria	Human	1	50	45	37(7)	25(4)	12(1)	12	2(1)
		Chimpanzee				NOT DONE				
		Baboon	0	61	61	61	61	59	59	59
June '77	New Jersey	Human	34(1)	17(3)	4(1)	9(3)	5	5(2)	3	3(3)
		Chimpanzee				NOT DONE				
		Baboon	58	1						
	Victoria	Human	0	2	2	1(1)				
		Chimpanzee	0	16	11	8	6	4	1	1
		Baboon	0	22	20	17	14	10	5	5
	New Jersey	Human	0	1	1	1	1	1	1	1(1)
		Chimpanzee	16							
		Baboon	21	1						

\* Number of individuals at indicated titer receiving vaccine.

employed throughout the study. Sera were pretreated with heat (56°, 30 min), trypsin, and periodate, according to procedures previously described (10). Appropriate controls and antigen "back-titrations" were included with each test.

**Results.** The survey was conducted over a 10-month period starting in September 1976, and ending in June 1977. Each month, 10–50 randomly collected serum samples were simultaneously tested, with the results given in Table I. The results indicate that influenza infection (principally by a strain related to A/Victoria) occurred in this area. All three primate species evidenced some level of antibody to the Victoria A antigen. Late winter testing suggested a possible localized outbreak evidenced by high titers to this antigen in all three species. Clinical evidence and virus isolation studies in the community confirmed these serologic findings. Similarly, lack of antibody (generally) to the newly isolated New Jersey strain, except in vaccinated individuals, as well as lack of isolation of virus from the community, indicated that this strain did not occur in the San Antonio area. No attempt was made to ascertain the reason for the few seropositives to the New Jersey strain that were recorded.

**Discussion.** Influenza, experimental and natural, has been reported (11) in various species of nonhuman primates. Very little is

known about influenza in simians under natural conditions, but this is also true for other viruses (11). The data reported herein suggest that nonhuman primates, as reflected by chimpanzees and baboons, follow the serologic pattern to influenza virus developed in humans. A/Victoria virus was in the community, and the primate population reflected this. Similarly, there was no evidence for human infection with the A/New Jersey strain, and this, too, was supported by the serologic data. No attempt was made to determine any epidemiologic factors associated with the results, but two observations may have some relevance: (1) The animals are housed in "open" cages, permitting access to small wildlife and birds, and (b) exposure to staff, while minimized, does occur.

Periodically, we isolate influenza viruses from the colony of baboons (12). The source of these infections is unknown, but it has generally involved newly imported animals under surveillance in quarantine. The pattern of seroconversion noted at times suggests horizontal transmission from animal to animal. Horizontal transmission in baboons following experimental infection has been reported previously (6, 13). It has also been observed that the duration of virus excretion (approximately 20 days post inoculation) is somewhat longer than that generally observed in humans (13). These data do not suggest a poten-

tial reservoir but, more probably, a host reaction closely akin to that occurring in humans.

**Summary.** Nonhuman primate (chimpanzees and baboons) sera were compared with human sera for serological activity to influenza viruses A/Victoria A/3/75 (H3N2) and A/New Jersey 8/76 (Hsw<sub>1</sub>N<sub>1</sub>). The results obtained indicate that all three primates reacted similarly to the influenza virus that was present in this area (A/Victoria). The data suggested that the nonhuman primates are not a potential reservoir but react to infection as do humans.

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## Effect of Hemolyzed Blood on Reticuloendothelial Function and Susceptibility to Hemorrhagic Shock<sup>1</sup> (40361)

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Severe depression of reticuloendothelial system (RES) phagocytic function is considered to contribute to the deterioration of an organism during circulatory shock (1-4). One aspect of the data supporting this concept is the finding that the injection of various foreign colloids including colloidal carbon, thorotrast, saccharated iron oxide and gelatinized lipid emulsion will induce a period of RES depression or blockade which is associated with increased susceptibility to various forms of shock (5-9). Additionally, RES blockade has been shown to be associated with the depletion of a plasma opsonic  $\alpha$ -2-glycoprotein and the circulating levels of this opsonic protein have been implicated in the control of RES phagocytic function (3, 8, 10-12).

Few studies have been carried out using altered homologous material as a potentially blocking substance even though the RES avidly clears such material from the circulation (13). RES blockade induced with altered homologous material would represent a much less artificial condition than the use of foreign or inert colloidal material. The present study was carried out to determine if a blockade-like depression of RES phagocytic function and increased susceptibility to shock is induced following the RES clearance of homologous erythrocyte cellular debris. Additionally,  $\alpha$ -2-glycoprotein opsonic activity was measured to determine its potential role in this form of RES depression.

**Methods.** Male Sprague-Dawley rats weighing 250-300 g were used for all experiments. Blood to be hemolyzed was collected in a plastic heparinized syringe from animals under ether anesthesia. The blood was hemolyzed by freezing at  $-20^{\circ}$  for 30 min and rapid thawing and warming to  $37^{\circ}$ . Hemolyzed blood was injected over 1-2 min at a dose of 0.3 ml/100 g and control animals

received an equal volume of heparinized non-hemolyzed blood.

Animals receiving injections of hemolyzed or non-hemolyzed blood were anesthetized with sodium pentobarbital (30 mg/kg, iv) and a femoral artery was cannulated. The animals were heparinized (100 USP units/100 g) and colonic temperature was monitored and maintained at  $36-37^{\circ}$ . Arterial blood pressure was monitored throughout the experiments. Thirty minutes after the injection of hemolyzed or nonhemolyzed blood, phagocytic index was determined, or a blood sample was taken for the determination of plasma opsonic activity, or hemorrhagic shock was induced for the evaluation of shock susceptibility.

Hemolyzed blood was separated into a particulate stroma and soluble supernatant fraction by centrifugation at 2000g for 15 min. The stroma fraction was washed three times in isotonic saline and resuspended in sufficient saline to bring the volume to the original blood volume. This stroma preparation contained approximately 13.5 mg of stroma protein/ml as determined with the Lowry assay. Similarly, the supernatant fraction was diluted with sufficient saline to bring the volume to the original blood volume. The fractions were injected iv at a dose of 0.5 ml/100 g, into animals prepared as described above, and phagocytic index was determined 30 min after injection.

Erythrocytes and erythrocyte stroma were labelled with  $^{125}\text{I}$  using a slight modification of the method of Hynes (14). Washed erythrocytes were suspended in phosphate buffered saline (PBS) (pH 7.2) plus 5 mM glucose to a hematocrit of approximately 50%. Carrier free  $\text{Na}^{125}\text{I}$  was added to a final concentration of 400  $\mu\text{Ci/ml}$  and the reaction was started by the addition of 3.2 units/ml of lactoperoxidase (Boehringer Mannheim, E.C. 1.11.1.7) and 0.1 units/ml of glucose oxidase (Boehringer Mannheim, grade I, E.C. 1.1.3.4). The mixture was incubated for 30 min at  $37^{\circ}$ .

<sup>1</sup> This research was supported by USPHS Grant No. HL-18051.

ction was stopped by the addition of 0.9% NaI, and the cells were washed and resuspended in an equal volume of saline. Erythrocyte stroma was prepared as described above. The clearance rate of erythrocyte and erythrocyte stroma was determined following the iv injection into anesthetized heparinized rats by taking blood (0.1 ml) at 5 min intervals for 30 min, the distribution of the  $^{125}\text{I}$  determined in liver, spleen, lungs and spleen. Half-time was determined from semilogarithmic plots of blood radioactivity time.

Phagocytic index for the hemolyzed blood and unlabelled stroma was determined by the clearance rate of gelatinized lipid emulsion labelled with  $^{131}\text{I}$  triolein as previously described (11, 12). The gelatinized lipid emulsion was injected iv at a dose of 50  $\mu\text{g}$ . Sequential blood samples were taken every 5 min and the half-time determined from semilogarithmic plots of blood radioactivity against time. Phagocytic index was calculated from the formula:  $\text{phagocytic index} = 0.301/\text{half-time}$ , where .301 is the  $\log_{10}$  of 2 and half-time is expressed in min. Five minutes after the colloid injection, the distribution of the colloid in the liver, lungs and spleen was determined.

Opsonic activity ( $\alpha$ -2-glycoprotein) was determined using the rat liver phagocytosis assay as previously described (11, 12). The assay evaluates the plasma opsonic stimulation of phagocytosis of gelatinized lipid emulsion by rat liver slices *in vitro*. The liver slices were incubated for 30 min in the presence of heparin, 1 ml of plasma, 2 ml of Tris-phosphate buffer (pH 7.4) and  $^{131}\text{I}$  labelled gelatinized lipid emulsion. At the end of the incubation, the liver slices were evaluated for the presence of  $^{131}\text{I}$ . Opsonic activity was expressed as  $\mu\text{g}$  of emulsion phagocytized per 100 mg of liver tissue ( $\mu\text{g}/100\text{ mg}$ ). Each plasma sample was assayed in triplicate.

Hemorrhagic shock was induced as previously described (15) by withdrawing sufficient blood via a cannulated femoral artery to reduce the mean arterial blood pressure to 40 mm Hg within 10 min. The arterial blood pressure was then maintained at 40–45 mm Hg by withdrawing small volumes of

blood until the point of initial decompensation, that is, when it was first necessary to return some of the withdrawn blood to maintain the blood pressure. Shock susceptibility was evaluated on the basis of the duration of hypotension required to reach the point of initial decompensation and the maximum shed volume.

Data were statistically analyzed using the unpaired Student's *t* test, placing the confidence level at 95%. All data are expressed as the mean and standard error of the mean.

**Results.** Phagocytic index, determined 30 min following the injection of hemolyzed whole blood, was decreased 44.7% ( $P < 0.01$ ) compared with control animals injected with an equal volume of nonhemolyzed blood (Fig. 1). Evaluation of the distribution of the test colloid 5 min after colloid injection revealed a 30.7% decrease ( $P < .01$ ) in liver phagocytosis and no change in the colloid localization in the spleen and lungs.

Following the injection of the particulate stroma fraction of hemolyzed blood phagocytic index was decreased 41.4% when compared to the saline controls (Table I). The injection of the soluble supernatant fraction of hemolyzed blood had no effect on phagocytic index. Tissue distribution of the test colloid showed that hepatic phagocytosis was depressed 37.7% following stroma injection and was unchanged after injection of the supernatant fraction. Localization of the colloid in the spleen was not changed. The stroma injection was associated with an increase in lung colloid localization, however,

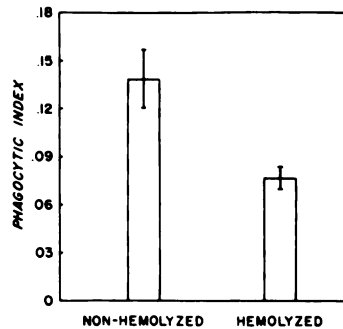


FIG. 1. Phagocytic index determined 30 min after the injection of hemolyzed or nonhemolyzed blood at a dosage of 0.3 ml/100 g. The values are different at  $P < .01$ . The values are expressed as mean  $\pm$  the SEM of eight animals per group.



the lungs contained only a small proportion of the injected colloid.

The intravenous injection of hemolyzed whole blood resulted in a large but transient decrease in arterial blood pressure. The rate of injection was adjusted so that the blood pressure was not reduced below 50 mm Hg which required that the blood be injected over 1–2 min. The pressure recovered to the preinjection level within  $2.3 \pm 0.3$  min after the start of injection. The pressure then increased to and remained at or above control levels for the remainder of the 30 min observation period. The injection of the supernatant fraction of hemolyzed blood resulted in a blood pressure response that was identical to that seen following the injection of whole blood. The injection of non-hemolyzed blood or the stroma fraction did not change arterial blood pressure.

Intact erythrocytes labelled with  $^{125}\text{I}$  were not cleared from the circulation at a sufficient rate to allow the determination of half-time over the 30 min observation period (Table II). The organ distribution of the erythrocytes is consistent with a very slow clearance rate. On the other hand, the erythrocyte stroma was rapidly removed from the circulation. A very substantial amount of the stroma was cleared by the liver with lesser amounts present in the spleen and lungs. This pattern of

particulate clearance is very similar to that observed with the test colloid clearance (Table I). The minimal amount of labelled erythrocyte stroma present in the kidney indicates little non-specific trapping in vascular beds.

Plasma opsonic activity determined 30 min after the injection of hemolyzed blood is presented in Table III. No differences were observed in plasma opsonic activity in animals injected with hemolyzed whole blood when compared with animals injected with non-hemolyzed blood.

Evaluation of the response to hemorrhagic shock revealed that the time to initial decompensation during hypotension was decreased 50.1% ( $P < .01$ ) in the animals injected with hemolyzed blood 30 min before initiation of hemorrhage (Fig. 2). There was no difference in maximum shed volume in animals injected with hemolyzed or nonhemolyzed blood. The large decrease in time to initial decompensation is interpreted as indicating an increased susceptibility to hemorrhagic shock in animals injected with hemolyzed blood.

**Discussion.** The present study has demonstrated that the injection of hemolyzed whole blood results in a large decrease in phagocytic index. This depression of RES phagocytic function was associated with a large reduction in the test colloid localization in the liver, with no change in the spleen and lung local-

TABLE I. PHAGOCYTIC INDEX AND ORGAN LOCALIZATION OF TEST COLLOID 30 MIN FOLLOWING INJECTION OF HEMOLYZED BLOOD STROMA OR SUPERNATANT FRACTIONS.<sup>a,b</sup>

	Phagocytic index (K)	Liver (%ID/TO)	Spleen (%ID/TO)	Lungs (%ID/TO)
Sham (saline)	.0947 $\pm$ .0070 <sup>c</sup>	46.10 $\pm$ 2.26	2.88 $\pm$ 0.31	0.75 $\pm$ 0.08
Stroma	.0555 $\pm$ .0086 <sup>d</sup>	28.70 $\pm$ 3.80 <sup>d</sup>	3.09 $\pm$ 0.29	1.28 $\pm$ 0.16 <sup>d</sup>
Supernatant	.0942 $\pm$ .0167	43.96 $\pm$ 4.03	2.39 $\pm$ 0.22	0.73 $\pm$ 0.06

<sup>a</sup> Stroma and supernatant fraction injected volume was 0.5 ml/100 g.

<sup>b</sup> Colloid localization was determined 5 min after injection of 50 mg/100 g  $^{131}\text{I}$  labelled gelatinized lipid emulsion and is expressed as the percent of the injected dose per total organ (%ID/TO).

<sup>c</sup> Values expressed as mean  $\pm$  SE;  $n = 7$ –10 for all groups.

<sup>d</sup>  $P < .01$  compared with the sham group.

TABLE II. CLEARANCE RATE AND ORGAN LOCALIZATION OF LABELLED ERYTHROCYTES AND ERYTHROCYTE STROMA.<sup>a</sup>

	Half-time (min)	Liver (%ID/TO)	Spleen (%ID/TO)	Lungs (%ID/TO)	Kidneys (%ID/TO)
Erythrocytes	— <sup>b</sup>	6.0 $\pm$ 0.4	1.9 $\pm$ 0.2	3.1 $\pm$ 0.2	0.34 $\pm$ 0.01
Erythrocyte stroma	1.86 $\pm$ 0.16	71.5 $\pm$ 2.2	5.6 $\pm$ 0.8	8.1 $\pm$ 0.9	0.42 $\pm$ 0.03

<sup>a</sup> Organ distribution was determined 30 min after iv injection of 0.5 ml/100 g and expressed as the percentage of the injected dose per total organ.

<sup>b</sup> Erythrocyte clearance was too slow to estimate the half-time over the 30 min observation period.

### TABLE III. PLASMA OPSONIC ACTIVITY 30 MIN AFTER THE INJECTION OF HEMOLYZED BLOOD\*

	<i>n</i>	Plasma Opsonic Activity ( $\mu\text{g}/100 \text{ mg}^b$ )
hemolyzed blood	6	$268 \pm 17^c$
non-hemolyzed blood	6	$291 \pm 19$

\*Dose of hemolyzed and nonhemolyzed blood was 0.3 ml/100 g.

<sup>b</sup>Opsonic activity is expressed as  $\mu\text{g}$  of gelatinized stroma phagocytized per 100 mg hepatic tissue.

<sup>c</sup>Values are expressed as the mean  $\pm$  SE of the mean of 10 animals per group.

Since the bulk of the colloid cleared from the circulation was removed by the liver, the depression of RES clearance was due primarily to a reduction in hepatic phagocytic

function. The fraction of whole hemolyzed blood responsible for the depression of phagocytic function appears to be the particulate stroma fraction. The depression of phagocytic index due to stroma injection was associated with a pattern of tissue colloid localization which is similar to that observed following whole hemolyzed blood injection. Additionally, the pattern of colloid distribution in the animals injected with hemolyzed blood or erythrocyte stroma was similar to previously seen during RES depression following RES colloidal blockage (11). The solubilization of hemolyzed blood had no effect on RES function which indicates that hemoglobin may or may not be removed from the circulation by the hepatic phagocytic cells (16, 17), the presence of free hemoglobin in the circulation does not depress RES function.

In response to the fractions of hemolyzed blood, in addition to demonstrating that the depressing substance is present in the particulate stroma fraction, also showed that RES depression was independent of the individual components of whole hemolyzed blood. This indicates that the RES depression is not due to the vasoactive material decreasing the hepatic blood flow sufficiently to delay colloid delivery to the hepatic Kupffer

cells obtained from the clearance of labeled erythrocyte stroma suggests that the amount of stroma employed in this study was cleared by the RES. This is based on (a)

the rapid rate of clearance from the circulation; (b) organ localization pattern which is very similar to that of the test colloid; and (c) minimal localization in the kidneys. The dose of erythrocyte stroma which was used to evaluate stroma clearance characteristics was identical to the dose which depressed RES phagocytic function. The persistence of the labeled intact erythrocytes in the circulation indicates that the rapid clearance of the stroma was not due to an alteration of the membrane during the labelling process. Thus, the particulate erythrocyte stroma fraction of hemolyzed blood is rapidly cleared from the circulation by the RES, and appears to be responsible for a blockade-like depression of RES phagocytic function.

The animals used in the present experiments were heparinized in order to eliminate the procoagulant effects of hemolyzed blood (18). This was done because it has been shown that intravascular coagulation induced by the injection of thrombin is associated with a depression of RES phagocytic function (19). Additionally, the high clearance rate of the control animals can be attributed to the heparin because in our hands heparin increases the rate of gelatinized lipid emulsion clearance (20). Other investigators have found that heparin increases (21, 22) or decreases the rate of colloid clearance (23). However, heparin does not reverse RES blockade following the injection of gelatinized lipid emulsion (21).

While the depression of RES phagocytic

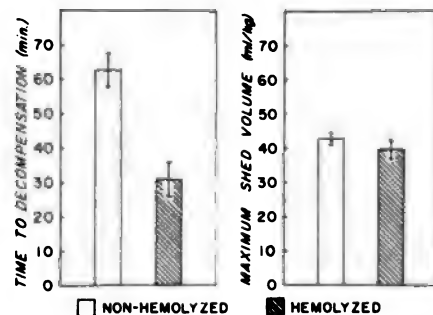


FIG. 2. Time to initial decompensation during hypotension and maximum shed volume in animals injected with hemolyzed and nonhemolyzed blood at a dosage of 0.3 ml/100 g. Hemorrhage was initiated 30 min after injection. Significant difference in time to decompensation ( $P < .01$ ). Values are expressed as the mean  $\pm$  SEM of 10 animals per group.

function following hemolyzed blood injection is similar to RES colloid blockade in terms of colloid clearance depression and the pattern of tissue colloid distribution, the lack of a depression of plasma opsonic activity is not consistent with the humoral opsonic factor theory of RES blockade. The depletion of plasma opsonic  $\alpha$ -2-glycoprotein activity from the circulation is a consistent finding with RES depression due to colloidal blockade (8, 10, 11) and various types of shock and injury (3, 4, 12, 15, 26). Since the RES depression associated with the injection of erythrocyte stroma is not associated with a depression of the circulating activity of this opsonic factor some other mechanism must mediate this RES depression. Such possible mechanisms may include the depletion of some other opsonic factor(s), saturation of phagocytic cell function or a decrease in liver blood flow of sufficient magnitude to limit delivery of the test colloid to the phagocytic cells. The data presented here suggested that a decrease in liver blood flow is not a likely mechanism.

The RES depression induced by the injection of hemolyzed blood was associated with an increased susceptibility to hemorrhagic shock. Since the whole hemolyzed blood contained a vasoactive component, and soluble proteins as well as stroma it is possible that the observed increase in shock susceptibility was not entirely due to the stroma-induced RES depression. Previous work by Hardaway *et al.* has shown that the injection of a small volume of hemolyzed blood into heparinized dogs resulted in an increased mortality with hemorrhagic shock (27). The present study suggests that this increase in mortality was due, in part, to a depression of RES phagocytic function. This notion is consistent with previous studies that have demonstrated that RES blockade with foreign material increased susceptibility to shock induced by hemorrhage, trauma, intestinal ischemia and endotoxin (5-9). Other studies by Subramanian *et al.* showed that intravascular hemolysis associated with experimental cardio-pulmonary bypass was associated with a depression of RES phagocytic function in terms of the clearance of colloidal gold and bacteria (28, 29). Thus, it is possible that hemolysis associated with severe burn or traumatic injury (30, 31) may contribute to RES depression

and thereby increase the rate of deterioration of the organism during shock.

**Summary.** RES phagocytic function and susceptibility to hemorrhagic shock were determined following the injection of hemolyzed blood into heparinized rats. Phagocytic index was severely depressed 30 min following the iv injection of whole hemolyzed blood (0.3 ml/100 g) and was due primarily to an impairment of hepatic phagocytosis of the test colloid. The erythrocyte stroma fraction of hemolyzed blood depressed phagocytic index while the soluble protein fraction had no effect on phagocytic index. Labelled erythrocyte stroma was rapidly cleared from the circulation and localized primarily in the liver with lesser amounts in the spleen and lungs indicating RES clearance of this particulate material. This depression of phagocytic index was associated with normal circulating levels of  $\alpha$ -2-glycoprotein opsonic activity. Animals injected with hemolyzed blood showed a 50% decrease in the duration of hypotension required to cause initial decompensation indicating an increased susceptibility to hemorrhagic shock. It is concluded that the hemolysis which accompanied severe injury such as burn or trauma may contribute to RES depression and increased susceptibility to shock states.

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## High Dosage of Testosterone Propionate Increases Litter Production of the Genetically Obese Male Zucker Rat (40362)

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Since it was first described in 1961 (1) the genetically obese Zucker rat has been of considerable interest as a possible animal model of human obesity, particularly that of early-onset. Homozygous recessive (*fafa*) individuals become recognizably obese near the time of weaning and are hypercellular (2), hyperinsulinemic (3) and hypertriglyceridemic (4). In addition to their weight regulatory dysfunction, *fafa* rats are reproductively inadequate. Obese sires are rare (1), and obese dams have not been reported. Virtually all *fafa* individuals have been derived from heterozygous (*Fafa*) crosses with an expected yield of 25%. Since *Fafa* and homozygous dominant (*FaFa*) individuals are phenotypically indistinguishable, obligatory testcrossing contributes to the inefficiency of production of the *fafa* genotype. The difficulty of obtaining adequate numbers of experimental subjects has so severely restricted work on the *fafa* rat that any improvement in the efficiency of its production would be welcome.

Factors predisposing for reproductive failure in the *fafa* rat have not been identified, but work on the *fafa* rat (5) and several studies on various genetically obese strains of mice (6-8) suggest steroid insufficiency as a proximal cause of abnormal reproductive morphology and low fertility. In our breeding colony at Vassar College we have been investigating the efficacy of steroid therapy in bringing about an improved breeding performance of intact *fafa* males. Subcutaneous injection of testosterone at high dosage levels showed promise. We report below experimental confirmation of the efficacy of this treatment in substantially enhancing the fertility of *fafa* males, its suppressive effects on increments in body weight and observations on the size and genotypic composition of litters resulting from the crossing of *fafa* males with known *Fafa* females.

**Materials and methods.** Twenty-eight *fafa* males ranging from 83 to 106 days of age

were randomly assigned to one of two treatment groups. Fourteen males received a subcutaneous injection of 20 mg testosterone propionate (TP) in 0.1 cc sesame oil for the first three consecutive days and 20 mg TP once every three days thereafter. Fourteen *fafa* males received sham injections of sesame oil on an identical schedule. Injections were continued over a 90-day period. On the third day of the experiment, two *Fafa* females were introduced to each male's cage and remained for 13 days whereupon they were removed and replaced by two other females. Thereafter, new *Fafa* females were provided each male every seven days. Thus during the 90-day experimental period each male had exposure to 24 females. Care was taken to assure that one of the females was a proven breeder whenever feasible, as we believed previous experience on the part of the female might improve the chance of impregnation. Females varied in age from three to 16 months. Males in both treatment groups were periodically weighed to detect any influence of TP on body weight.

**Results.** The numbers of litters sired by the two groups of males during the 90-day experimental period are summarized in Table 1. The difference in production is substantial: TP-injected males sired 73 litters while sham-injected males sired 19 ( $P < .001$ , Chi-square test). This disparity in litter production by the two treatment groups is attributable to three factors. Eleven TP males became sexually active compared to seven sham-injected males. Mean latency to first conception for sexually active TP males was 15.9 days (range 2-36 days); for active sham-injected males: 24.1 days (range 2-56 days). The rate of impregnation was higher for active TP than active sham males: 30.6% of females placed with TP males after they had sired their first litter gave birth whereas only 10.5% of females placed with proven sham males bore young. For comparison, 90.2% of females

placed with eleven similarly experienced non-injected *Fafa* males in an otherwise identical breeding regimen conceived. Females with prior breeding experience were no more likely than inexperienced females to conceive when placed with TP or sham males.

The breakdown of litter conception into consecutive 30-day periods (Table I) reveals a sharp drop in the number of males active and the number of litters sired for both treatment groups during the last third of the treatment period. While the number of litters remained significantly higher ( $P < .01$ , Chi-square test) for the TP males, it appears that the efficacy of TP attenuates with time. A separate experiment in which thirteen *fafa* males seven to eleven months of age received 20 or 30 mg TP ( $n = 11$ ) or sham ( $n = 2$ ) for 90 days in the regimen described above resulted in no litters. Females were provided to these older males in the same manner as for young males.

Table II provides information which makes possible a comparison of the size and composition of litters from *fafa* and *Fafa* males paired with *Fafa* females. Size and composition of litters from *FaFa*  $\times$  *FaFa* crosses are included for comparison. Litter size at birth did not differ significantly among groups, nor did litter size at weaning. For *Fafa* sired litters the *fafa* pups comprised 25.5% of the offspring, which conforms to expectation. In *fafa* sired litters 44.5% of the pups were *fafa*. This is a significant departure from the expected 50% ( $P < .05$ , Chi-square test). Between birth and weaning *fafa* sired pups exhibit a 21.2% mortality, lean sired pups a 14.7% mortality. The difference is significant ( $P < .01$ , Chi-square test). Among lean pups and obese pups, regardless of parentage, there

is a slightly smaller number of male pups than female pups at weaning age.

At the beginning of the experiment, the young TP-injected males had a mean body weight of  $354 \pm 6.7$  g<sup>1</sup>; the young sham-injected males  $334 \pm 13.6$  g. The difference was not statistically significant. On day 89 of treatment the mean weight of TP males was  $505 \pm 13.8$  g; sham males  $584 \pm 16.2$  g ( $P < .001$ ,  $t$  test). Changes in body weight with time are shown in Fig. 1 as mean percent increase over initial body weight. The rate of weight gain was significantly reduced ( $P < .01$ ,  $t$  test) as early as 29 days after treatment was begun.

Partial correlational analyses of litter production and body weight dynamics among the TP-injected males revealed no significant association between either latency to first conception or number of litters sired and initial body weight, final body weight, the changes in body weight or the percent increase in body weight. The same was true for sham-injected males.

**Discussion.** High doses of testosterone propionate clearly increase the litter production of the young *fafa* male rat. TP-injected males sired nearly four times as many litters as sham-injected controls. The improved litter production makes practical the use of *fafa* males instead of heterozygous males for breeding with heterozygous females. This should increase greatly the efficiency of producing *fafa* rats since nearly twice as many will result from a successful mating. The breeding of *fafa* males with lean females also guarantees that any phenotypically lean offspring are heterozygous. Thus, testcrossing to identify heterozygous rats is no longer necessary.

The suppressive effect of TP on rate of weight gain is attributable, at least in part, to reduced food consumption. We have preliminary data which indicate that *fafa* males given TP in the same regimen as in our breeding experiment significantly reduce their daily food intake.

The possibility that the increased obesity which accrues with age contributes to the reproductive impairment of *fafa* males is suggested by the sharp decline in litter production in both TP-and sham-injected young

TABLE I. EFFECT OF TESTOSTERONE PROPIONATE ON THE LITTER PRODUCTION OF YOUNG *fafa* MALES.

Treatment	Number of males	Numbers of litters conceived			
		0-90 days	1-30 days	31-60 days	61-90 days
TP <sup>a</sup>	14	73 (11) <sup>b</sup>	25 (9)	32 (11)	16 (7)
Sham	14	19 (7)	8 (5)	9 (5)	2 (2)

<sup>a</sup> 20 mg testosterone propionate in 0.1 cc sesame oil administered subcutaneously once every three days.

<sup>b</sup> Numbers in parentheses indicate the number of males responsible for the litters conceived during the above indicated span of time.

<sup>1</sup> S.E.M.

TABLE II. SIZE AND COMPOSITION OF LITTERS Sired BY *fafa*, *Fafa* AND *FaFa* MALES.

Genotype of parents		Mean litter size			Phenotype and sex			
Male	Female	Number of litters	At birth	At weaning	Lean (Fa—)		Obese ( <i>fafa</i> )	
					Male	Female	Male	Female
<i>fafa</i> (19) <sup>a</sup>	<i>Fafa</i> (49)	55	8.78 ±.41 <sup>b</sup>	6.92 +.51	102 55.4% <sup>d</sup>	110	78 44.6%	93
<i>Fafa</i> (30)	<i>Fafa</i> (50)	55	9.40 ±.42	8.06 ±.42	159 74.5%	171	54 25.5%	59
<i>FaFa</i> (16)	<i>FaFa</i> (46)	55	9.02 ±.47	7.57 <sup>c</sup> ±.79	70 <sup>c</sup> 100%	79 <sup>c</sup>		

<sup>a</sup> Numbers in parentheses indicate the number of individuals of this type involved in the production of the litters on which the data is based.

<sup>b</sup> S.E.M.

<sup>c</sup> Data based on 21 litters. Remainder of those used for determining litter size at birth were utilized in experiments before weaning age.

<sup>d</sup> Frequency of phenotypes at weaning expressed as a percentage.

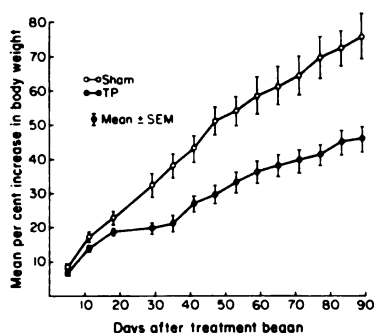


FIG. 1. Effect of testosterone propionate on the rate of weight gain in young *fafa* males.

males toward the end of the testing period and the total lack of response of the older, more obese, males to treatment. Hemmes and Hirsch (9) have recently reported that Osborne Mendel rats rendered obese by feeding a high fat diet exhibit markedly diminished sexual vigor. These findings together with the observation that substantially reducing the weight of *fafa* males improves their litter production (P. Johnson, personal communication) lead us to suspect that factors secondary to the obese condition contribute to the infertility of *fafa* males.

The efficacy of TP in increasing litter production suggests that *fafa* males may have a testosterone deficiency. Circulating levels of testosterone have not been reported for the Zucker rat. Testosterone deficiency is known to occur in morbidly obese men. Glass *et al.* (10) have suggested that aromatization of testosterone by the enlarged adipose depot

may be responsible for the deficiency. Barbato and Landau (11) report that, after substantial weight loss, testosterone levels of obese men return to the normal range and that sexual performance and libido improve. Further study would reveal the extent to which adipose tissue, steroid levels, and reproductive function are causally interrelated.

**Summary.** A high dose of testosterone propionate increases dramatically the litter production of young genetically obese male Zucker rats. Twenty milligrams testosterone injected subcutaneously once every three days over a 90-day period resulted in a nearly fourfold increase in the number of litters sired compared to sham-injected controls. The efficacy of the treatment attenuates with time. TP was ineffective in inducing litter production in older, more obese, males. Young obese males injected with TP exhibited a significantly reduced rate of weight gain compared to sham-injected controls. The findings are consistent with the hypothesis that the reproductive inadequacy of the genetically obese male rat may be due to a deficiency of circulating testosterone. The treatment of obese males with TP greatly increases the efficiency with which the obese (*fafa*) genotype may be produced and also avoids time-consuming testcrossing for identification of heterozygous (*Fafa*) individuals.

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## Effect of Kidney Surface Temperature on Single Nephron Filtration Rate (40363)

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In 1970, McDonald and Sparks (1) reported in a preliminary communication that blood flow to the decapsulated area prepared for micropuncture appeared to be slower than was flow to the superficial cortex in the normal dog kidney with an intact capsule. Shortly afterward, Clapp and his associates (2, 3) suggested that nephron function was significantly improved during dog micropuncture studies, if the exteriorized kidney was wrapped in saline-soaked sponges and insulated against heat loss by an "overall covering wrap of clear and light weight plastic." Kidney surface temperature was well maintained at 37° with these protective features but fell promptly to 35° when the plastic wrap was not utilized. Later, Deetjen and Silbernagl (4), reported that a decrease in whole body temperature of rats is accompanied by a decrease in both cardiac output and renal cortical blood flow; mean arterial blood pressure (BP) remained constant. Extrapolation from their data at both 37° and 35° suggests that the magnitude of the decrease in outer cortical blood flow was about 30%.

Taken together, these observations imply that an exteriorized kidney prepared for micropuncture studies might function at levels that are below normal, possibly to a greater degree in the decapsulated area. Thus, reduced nephron blood flow and/or filtration rate at the micropuncture site might occur. Whole kidney clearance measurements however, might not reflect this local diminution in function. The current studies were designed to reevaluate the reports of Clapp (2, 3) and to quantitate any improvement in single nephron glomerular filtration rate (SNGFR) that may accompany the preservation of surface temperature at a near normal value.

**Methods.** Mongrel dogs of both sexes weighing 18-26 kg were anesthetized with sodium pentobarbital (30 mg/kg, iv) and intubated with a cuffed endotracheal tube. Peripheral catheters were placed in superficial

veins to infuse inulin and para-aminohippurate (PAH) at 1.0 ml/min and a maintenance infusion of isotonic saline at 2.0 ml/min. A catheter was placed in each femoral artery, one to measure mean arterial blood pressure (MABP) with a Harvard transducer (model 377) and to collect blood samples. The other catheter was advanced into the aorta and its tip positioned just above the left renal artery. Small volumes (0.5-1.0 ml) or 10% lissamine green dye were injected through this catheter to visualize proximal and distal tubules. The right ureter was catheterized via a suprapubic incision.

Via a flank incision, the left kidney, renal artery and vein were exposed. The left renal vein was catheterized from a gonadal vein to quantitate PAH extraction. In addition, a flow probe (Carolina Medical Electronics, Inc., King, NC) placed on the left renal artery permitted direct measurement of renal blood flow (RBF) at endogenous MABP. An adjustable brass clamp was placed on the aorta above the left renal artery in order to reduce renal perfusion pressure to determine the RBF autoregulatory capability of the kidney (5-9). This test was performed after completion of surgery. All experiments were conducted at endogenous MABP which was above the lower limit of autoregulation. Finally the left ureter was catheterized near the hilus.

The left kidney was mounted in a plastic cup attached to a steel micropuncture table above the dog. A small 1-2 cm<sup>2</sup> area of capsule was removed from the surface of the mounted kidney in order to visualize tubules. Warm (37°) oil was dripped on the decapsulated surface. A fiberoptic (Dolan-Jenner Industries, Inc.) was used to illuminate the micropuncture field. A small (1.0 mm diameter) thermistor connected to an electronic thermometer was placed on the exposed surface near the border between the decapsulated and intact capsule to monitor kidney surface temperature. At this point the dogs were di-

d into two groups. In the first (group I; 10) the exteriorized kidney was covered with warm saline-soaked sponges and covered with a loose insulating plastic wrap to prevent heat loss and evaporation from the exposed organ (Fig. 1). Micropuncture was performed through a small "window" in the plastic wrap. Three to four collections from individual nephrons and as many distal collections as possible were obtained during the study. Each tubular fluid (TF) collection was followed immediately by a collection of arterial blood in order to measure plasma inulin concentration; collections from proximal or distal nephrons were obtained randomly. The micropuncture collection period which lasted about one hour was begun sixty minutes after placement of the kidney in the cup and initiating the infusion of inulin and PAH. During the micropuncture study, two 30-min collections of tubular fluid were obtained from each kidney along with mid-point arterial and renal vein blood samples. In group II ( $n = 9$ ) the kidney was wrapped. Urine and tubular fluid collections were obtained with a protocol that essentially was identical to group I.

**Analytic methods.** Plasma (P) and urine (U) inulin and PAH were both measured by autoanalyzer technique (10). Hematocrit was measured by microcentrifugation. Tubular fluid (TF) inulin concentration was determined by the fluorometric method of

Vurek and Pegram (11). SNGFR was calculated from the formula

$$\frac{TF_{In}}{P_{In}} \times \dot{V} \text{ (nl/min)} = \text{SNGFR (nl/min)}$$

where  $\dot{V}$  is the quantitative collection rate of TF expressed in nl/min.  $\dot{V}$  in nl was measured with a constant bore capillary tube. Standard clearance formula was used to calculate inulin and PAH clearance. Renal plasma flow (RPF) was estimated by both PAH clearance and extraction and by flowmeter estimates of RBF and hematocrit measurements in most experiments. In an occasional dog, two renal arteries prevented the use of the flow probe; however when both techniques were used simultaneously, estimates of RBF agreed to within 4% in any single experiment. All measurements of RPF and RBF reported in this study are based upon clearance and extraction of PAH. At the end of each experiment kidneys were removed, blotted dry, decapsulated and weighed. Standard statistical techniques (paired and unpaired  $t$  test) were used to determine significant differences. Individual SNGFR values were averaged to provide a single mean value for each site (proximal or distal) from each dog. Values are mean  $\pm$  one SE.

**Results. Renal clearance and hemodynamic measurements.** Table I demonstrates there were no significant differences in either inulin



1. The clear plastic wrap (overlying the warm saline soaked sponges) covering the entire kidney is shown. Tape secures the wrap to the kidney holder and is stretched over the kidney and secured to the sides of the micropuncture table. Micropuncture is performed through a small "window" in the plastic wrap. The decapsulated site indicated by the arrow and approximately 1 cm<sup>2</sup>.

clearance, RPF, RBF or MABP among the two groups. However, temperature at the surface of the kidney was consistently and significantly lower ( $P < 0.002$ ) when the plastic wrap was omitted.

**Micropuncture-group I** (Fig. 2). In 10 dogs, proximal SNGFR averaged  $72 \pm 7$  nl/min (range: 46–108 nl/min) and significantly ( $P < .01$ ) exceeded distal SNGFR which averaged  $46 \pm 4$  nl/min (range: 23–68 nl/min). Distal  $\dot{V}$  averaged  $14.8 \pm 1.8$  nl/min and  $TF/P_{in}$  averaged  $3.37 \pm 0.32$ . MABP averaged  $112 \pm 7$  mm Hg (range: 80–140 mm Hg).

TABLE I. RENAL FUNCTION, MEAN ARTERIAL BLOOD PRESSURE AND KIDNEY SURFACE TEMPERATURE.

	$C_{in}$	RPF <sup>a</sup> (ml/min · g KW)	RBF <sup>a</sup>	MABP (mm Hg)	Temp. °C
Group I (n = 10)					
W	0.57 <sup>b</sup> ±0.06	1.99 ±0.16	3.70 ±0.24	113 ±7	37.6 ±0.1
Group II (n = 9)					
U	0.58 ±0.09	2.66 ±0.29	4.40 ±0.50	112 ±7	35.7 ±0.1
P	>.9	>.05	>.2	>.6	<.002

<sup>a</sup> RPF was determined by PAH clearance and extraction; RBF was determined from RPF and hematocrit. Flow rates are ml/min · gram kidney weight.

<sup>b</sup> Mean ± SE;  $C_{in}$  = inulin clearance; RPF = renal plasma flow; RBF = renal blood flow; MABP = mean arterial blood pressure; Temp. = surface temperature in °C of kidney prepared for micropuncture; U = unwrapped; W = wrapped.

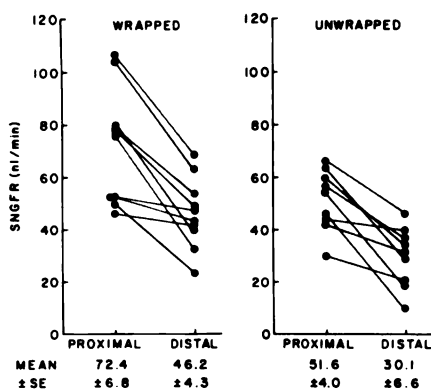


FIG. 2. Comparison of individual proximal and distal SNGFR values from 10 dogs in which the kidney was wrapped with plastic (WRAPPED) and from nine dogs in which the plastic wrap (UNWRAPPED) was not used. Blood pressure, GFR and RBF were similar between the two groups (see Table I).

**Micropuncture-group II** (Fig. 2). As in group I, proximal SNGFR also significantly ( $P < .02$ ) exceeded distal SNGFR. In nine dogs, proximal SNGFR averaged  $52 \pm 4$  nl/min (range: 29–66 nl/min) which was significantly lower ( $P < .02$ ) than average proximal SNGFR in wrapped kidneys (group I). The average distal SNGFR of  $30 \pm 7$  nl/min (range: 10–46 nl/min) was also significantly lower ( $P < .02$ ) than that of wrapped kidneys (group I). Distal  $\dot{V}$  was lower by more than 50% averaging  $6.1 \pm 1.0$  nl/min ( $P < .001$ ) and  $TF/P_{in}$  was higher averaging  $5.43 \pm 0.48$  ( $P < .01$ ). MABP was similar to group I averaging  $113 \pm 7$  mm Hg (range: 84–150 mm Hg).

**Discussion.** The results of these studies suggest the exteriorized wrapped kidney prepared for micropuncture maintains a more normal surface temperature and the nephrons demonstrate higher values for both proximal and distal SNGFR compared to the unwrapped kidney. Cooling of the outer kidney surface by exposure to room temperature might induce the type of vasoconstriction characteristic of other vascular beds exposed to cold (12). The effects of cooling could be more pronounced in the area of micropuncture where the capsule has been removed as has been suggested by McDonald and Sparks (1). The apparent local reduction in flow in that study was not accompanied by measurable changes in whole kidney function which is consistent with the present observations. Moreover, these results suggest also that only a small region of the exteriorized kidney could be significantly influenced by exposure to room temperature and overall renal hemodynamics including RBF might remain within normal limits. However, any substantial RBF decrease at the area prepared for micropuncture could well proved an appropriate explanation for the lower SNGFR we have observed. The lower surface temperature (2, present study), apparent decreased local blood flow (1), and lower proximal and distal SNGFR (present study) all suggest that diminution in nephron function might occur in the decapsulated area if appropriate caution is not taken in preparing the kidney for micropuncture studies.

Clapp *et al.* (2, 3) have reported a similar qualitative interpretation of nephron function in the wrapped versus the unwrapped kidney.

Although providing no quantitative data, these investigators report "renal function was more improved following wrapping of the kidney." Our present data provide some measure of the improvement induced in the wrapped kidney. Distal SNGFR averaged about 30 nl/min in unwrapped kidneys which is approximately 35% lower than the average dog distal SNGFR (44–47 nl/min) reported for wrapped kidneys (8, present study).

Finally, the difference between proximal and distal SNGFR in the same kidney whether wrapped or unwrapped, appears to indicate that orthograde flow to the macula densa is an important factor which regulates afferent arteriolar tone and thus SNGFR (8, 9, 13). The data also suggest that a tubuloglomerular feedback system sensitive to changes in "distal delivery" (13) does exist and can be demonstrated even in unwrapped kidneys, where proximal SNGFR exceeds distal SNGFR by about 21 nl/min. However, the reduced surface temperature might have led to local vasoconstriction thereby impairing assessment of normal distal and proximal nephron function in the dog.

In conclusion, the results of these studies indicate that an improvement in nephron function does occur in the exteriorized kidney which is protected against exposure to room temperatures. Quantitatively, superficial single nephron glomerular filtration rate (SNGFR) measured at distal nephron sites averaged about 35% less in unwrapped kidneys (surface temperature average 35.7°) compared to similar studies in wrapped kidneys (surface temperature average 37.6°); whole kidney GFR, RBF and BP were similar in both studies. When surface temperature is maintained by an insulating plastic wrap, we agree with Clapp and coworkers (2, 3) that "... stability of function was significantly improved ...".

**Summary.** In dog kidneys prepared for micropuncture experiments, the thesis that exteriorized organs with an intact circulation may demonstrate reduced function due to exposure to cool (21–23°) room temperatures, was tested by measuring superficial proximal and distal SNGFR on the surface of kidneys either protected against heat loss with a plastic wrap or unwrapped and exposed to room temperature. No significant differences in

GFR or RBF could be detected between these conditions. However, temperature at the kidney surface was 37° in wrapped kidneys but fell ( $P < .002$ ) to 35° in the unwrapped state. The lower surface temperature was associated with reduced values for proximal SNGFR,  $72 \pm 7$  vs.  $52 \pm 4$  nl/min ( $P < .02$ ) and distal SNGFR,  $46 \pm 4$  vs.  $30 \pm 7$  nl/min ( $P < .02$ ). The results indicate that the uninsulated kidney prepared for micropuncture may have decidedly lower values for superficial SNGFR measured by total collections of tubular fluid from either proximal or distal sites. These data also suggest that the reductions may be local because whole kidney function does not indicate a similar quantitative reduction in function.

Portions of this study have been reported (Fed. Proc., 35: 541, 1976). Drs. Duchin and Peterson are postdoctoral trainees supported, as was this research, by a USPHS Grant No. AM 17646. Ms. Susan J. Christie and Ms. Carole S. Bucher provided excellent technical and secretarial support, respectively. We thank Dr. Robert W. Schrier for his advice and suggestions during this study and the preparation of this manuscript.

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# Blood Pressure Responses to Extremes of Sodium Intake in Normal Man<sup>1</sup> (40364)

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Although a connection between dietary salt intake and the development of hypertension has been proposed by many observers, the evidence presently available is largely circumstantial (1). Increases in blood pressure have been observed with increases in salt intake in subjects with diminished renal function since the classic report of Ambard and Beaujard in 1904 (2); however, reports of increasing blood pressure with increasing salt intake in normal subjects have been few and anecdotal (3, 4).

Guyton and associates (5) have developed a systems analysis approach which provides a conceptual framework for integrating the various mechanisms that control blood pressure. Their analysis suggests that the kidney's ability to excrete salt and water is the overriding mechanism of blood pressure regulation. They termed the relationship between the state of salt balance and blood pressure the renal function curve. The renal function curve indicates the blood pressure for any state of salt balance in the intact organism. Alterations in the renal function curve may be important in the generation of chronic hypertension in man. We have undertaken studies in normal men and have observed increases in blood pressure with extremes of salt intake. Our data describes the relationship between the kidney's ability to excrete salt and water and the systemic blood pressure.

**Methods.** Eight normotensive, healthy

male volunteers (mean age 32 years, range 22-40) were obtained by advertisement and were studied at the Indiana University Clinical Research Center. The protocol was approved by the Indiana University Medical Center Human Use and Clinical Research Center Committees and informed consent was obtained from each volunteer after detailed explanation of the procedures to be performed.

**Protocol.** The subjects were given a constant diet containing 10 mEq sodium, 80 mEq potassium, 65 gms protein, 50 gms fat, 270 gms carbohydrates, 400 mg calcium and 1000 mg phosphorus daily. Dietary sodium intake was maintained at 10 mEq for seven days, 290 mEq of sodium in the form of sodium chloride were added to the diet for 3 days (300 mEq sodium diet), and 790 mEq sodium were added to the diet for 6 days (800 mEq sodium diet). In order to achieve an 800 mEq sodium intake, sodium was given with bouillon between meals and at bed time. All meals were eaten in the Clinical Research Center; however, the subjects were not hospitalized until the final three days of the study when they received an additional 700 mEq sodium in the form of intravenous normal saline throughout the night. The design of the study was such that the subjects received 10 mEq Na/24 hr for 7 days, 300 mEq Na/24 hr for 3 days, 800 mEq Na/24 hr for 3 days, and 1500 mEq Na/24 hr for 3 days. Fluid intake (distilled water) was allowed *ad libitum*.

The subjects were weighed every morning before breakfast after voiding. Blood pressures were obtained daily before meals by the indirect auscultatory technique. The same mercury manometers (Baum, Inc., New York, NY) and the same cuffs were employed throughout the study. The subjects rested supine in a darkened room for 5 min after which blood pressure and measurements of heart rate were obtained in the nondominant

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arm each minute for 5 min. The same two observers (RB and FL) were responsible for these measurements throughout the study. Mean arterial blood pressure was calculated by adding one-third the pulse pressure to the diastolic pressure.

Twenty-four hour urine specimens were obtained daily for the determination of sodium, potassium, and creatinine concentrations. At 7:00 AM on the morning of the final day at each level of sodium intake, blood specimens were obtained following two hours of ambulation for hematocrit, creatinine, sodium, potassium, plasma renin activity, and plasma aldosterone concentrations. Stroke volume and cardiac output were measured noninvasively by echocardiography on the final day at each level of sodium intake (6). The echocardiograms were interpreted by two observers without knowledge of the regimens.

**Subject safeguards.** To tolerability of the diet was examined in an initial pilot study. Two of the investigators (RB and FL) and a medical student volunteer ingested first the 10 mEq/day sodium diet for 1 week, followed by the 800 mEq/day sodium diet for one week. We found that the diet was tolerable and that a generous intake of free water eliminated the tendency to develop diarrhea at the higher sodium loads. Additional sodium was also infused intravenously to total 1200 mEq/day and no ill effects were noted. Balance was achieved at the 10 mEq/day level by the 6th day in every subject. When sodium intake was increased to 300 mEq/day or higher, balance was approached by 72 hr.

Two of the eight subjects were physicians (RB and FL), one taught high school biology, and five were Indiana University Hospital employees. All were well aware of the nature and the potential risks of the study. The subjects were examined by a physician thrice daily except at the 1500 mEq/day sodium intake at which time they were examined four times daily. None developed any adverse symptoms other than the fatigue ostensibly related to the sleeplessness because of nocturia. At the 1500 mEq/day sodium intake, pedal edema became clinically detectable. No rales or gallop rhythms were heard in any subject; no electrocardiographic changes were observed. The chest roentgenograms revealed

a detectable increase in cardiac size in five subjects and small pleural effusions in two subjects at the end of the last study day. Three days after the experiment, the subjects' weights, blood pressures, and chest roentgenograms had returned entirely to normal, as had their sense of well being.

**Laboratory methods.** Sodium and potassium concentrations in plasma and urine were measured by a flame photometer (Instrumentation Laboratories, Boston, MA). Creatinine was measured by an automated technique (Technicon, Chauncey, NY). Plasma renin activity and plasma aldosterone were measured by previously reported radioimmunoassay methods (7). The data was analyzed statistically by two way repeated measures analysis of variance. A computerized program was employed.

**Results.** The variables obtained on the last day at each level of sodium intake were tabulated and are outlined in Table I. Increasing sodium intake had a significant effect on body weight ( $P < 0.001$ ), mean arterial blood pressure ( $P < 0.001$ ), sodium excretion ( $U_{Na}V$ ) ( $P < 0.001$ ), potassium excretion ( $U_KV$ ) ( $P < 0.001$ ), creatinine clearance ( $P < 0.025$ ), plasma renin activity (PRA) ( $P < 0.001$ ), plasma aldosterone concentration (PA) ( $P < 0.001$ ), stroke volume ( $P < 0.01$ ), and cardiac output ( $P < 0.025$ ). The heart rate remained unchanged.

Compared to the 10 mEq/day level of sodium intake, mean arterial blood pressure was significantly increased at the 800 mEq/day level ( $P < 0.05$ ), but not at the 300 mEq/day level. An additional increase ( $P < 0.01$ ) occurred between the 800 mEq/day and 1500 mEq/day levels of sodium intake. The relationship between systemic blood pressure and sodium excretion is graphically displayed in Fig. 1. The interaction between systolic, diastolic and mean blood pressure, and sodium excretion was highly significant ( $P < 0.001$ ).

The urinary potassium excretion and creatinine clearance were both increased significantly by the 800 mEq/day level of sodium intake ( $P < 0.05$ ). The increase in sodium intake to 1500 mEq/day resulted in another increase in kaliuresis ( $P < 0.01$ ); however, no further increase in creatinine clearance was observed ( $P > 0.05$ ). Consistent changes in

TABLE I. CHARACTERISTICS FOLLOWING BALANCE AT EACH LEVEL OF SODIUM INTAKE (MEAN  $\pm$  SD).

Sodium intake (mEq/24 hr)	10	300	800	1500
Weight (kg)	78.2 $\pm$ 12	79.5 $\pm$ 12	80.5 $\pm$ 12	83.1 $\pm$ 11
Mean blood pressure (mm Hg)	82.6 $\pm$ 6	83.8 $\pm$ 7	89.5 $\pm$ 8	99.2 $\pm$ 9
Heart rate (beats/min)	62 $\pm$ 12	63 $\pm$ 13	58 $\pm$ 9	60 $\pm$ 11
$U_{Na}V$ (mEq/24 hrs)	12 $\pm$ 4	265 $\pm$ 68	702 $\pm$ 67	1442 $\pm$ 100
$U_KV$ (mEq/24 hrs)	66 $\pm$ 18	74 $\pm$ 10	142 $\pm$ 20	182 $\pm$ 36
Plasma Na (mEq/L)	138.8 $\pm$ 4	139.5 $\pm$ 3	132.6 $\pm$ 6	135.6 $\pm$ 2
Plasma K (mEq/L)	3.7 $\pm$ .3	3.6 $\pm$ .2	3.9 $\pm$ .4	3.6 $\pm$ .2
Creatinine clearance (ml/min)	110 $\pm$ 23	126 $\pm$ 8	131 $\pm$ 22	137 $\pm$ 12
Plasma renin activity (ng AI/ml/3 hr)	13.5 $\pm$ 8.0	2.6 $\pm$ 2.0	1.3 $\pm$ 1.0	0.7 $\pm$ 0.4
Plasma aldosterone (ng/100 ml)	39 $\pm$ 22	9.0 $\pm$ 7.0	2.6 $\pm$ 2.0	1.6 $\pm$ 0.4
Stroke volume (ml/beat)	86 $\pm$ 12	93 $\pm$ 13	100 $\pm$ 11	115 $\pm$ 10
Cardiac output (L/min)	5.3 $\pm$ 0.5	5.9 $\pm$ 1.2	5.8 $\pm$ 1	6.9 $\pm$ 1.8

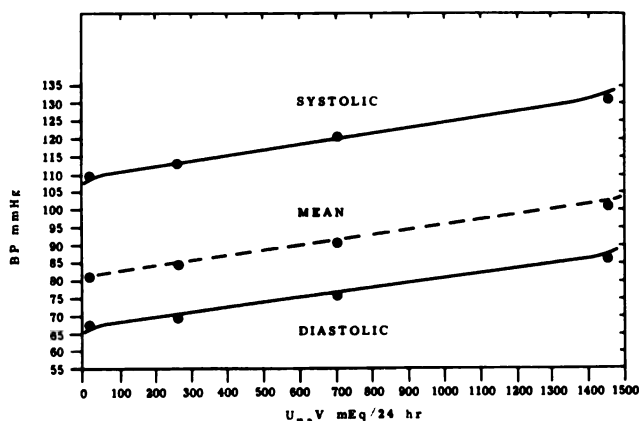


FIG. 1. The relationship between systolic, diastolic and mean arterial blood pressure and sodium excretion in eight normal subjects.

plasma sodium and plasma potassium concentration were not observed. The plasma sodium concentration obtained at the 800 mEq/day level of sodium intake differed from the two lower levels of sodium intake ( $P < 0.05$ ); however, there was no difference in mean plasma sodium concentration between the lowest and highest levels of sodium intake. The plasma potassium concentration at the 800 mEq/day level of sodium intake differed from the plasma potassium concentration at the 300 and 1500 mEq/day levels of sodium intake. Plasma renin activity and plasma aldosterone concentration decreased ( $P < 0.01$ ) between the 10 and 300 mEq/day levels of sodium intake. Stroke volume increased at the 800 mEq/day ( $P < 0.05$ ) and again at the 1500 mEq/day ( $P < 0.05$ ) levels of sodium intake. The cardiac output increased at the 1500 mEq/day level of sodium intake ( $P < 0.05$ ).

**Discussion.** These results demonstrate a relationship between the states of sodium balance and systemic blood pressure in our normotensive subjects which was similar to that predicted by Guyton's systems analysis (5). Although there was no apparent effect on systemic blood pressure by increasing sodium intake from 10 to 300 mEq/day, increases to 800 mEq/day and 1500 mEq/day resulted in a stepwise significant increase in systemic blood pressure. Kirkendall *et al.* (8) studied normotensive human volunteers after four week exposure to 10 mEq/day, 210 mEq/day and 410 mEq/day levels of sodium intake. They were unable to document an increase in systemic blood pressure in their subjects. Relman and Schwartz (9) studied normotensive volunteers under conditions of sodium intake up to 450 mEq/day. Their subjects received intramuscular injections of desoxy-corticosterone acetate. They observed

small slight elevations of arterial diastolic pressure in two of three subjects. These are consistent in that they suggest that the increases in sodium intake are necessary in normal subjects to effect an increase in systemic blood pressure.

Our data support much earlier observations that massive increases in sodium intake provoke an increase in systemic blood pressure. McDonough and Wilhelmj (3) gave 37 mEq sodium daily to a single normotensive subject for 23 days and observed a rise in systemic blood pressure. Facial edema also occurred in their patient. Marrie, Thompson, and Anderson (4) administered the adult equivalent (by body weight) of from 1204 to 2408 mEq of sodium to diabetic children. They noted 30% increases in both systolic and diastolic pressures above control values. Although the state of sodium balance was not entered in these early reports, and statistical analyses were not applied, they indicate that sodium may be associated with increases in systemic blood pressure if huge amounts are given.

The mean 24 hr urinary sodium excretion measurements obtained in our subjects at the different levels of sodium intake indicate that the state of sodium balance was approached at each level. The difference between ingested and excreted sodium in our study likely reflects fecal and cutaneous losses of the ion and does not reflect inadequate urine collection.

Urinary potassium excretion increased progressively with increasing sodium intake. Our findings were reported by Kirkendall *et al.* (8), who raised the possibility that the kaliuresis was engendered by physical displacement of potassium from intracellular spaces by sodium. The kaliuresis may also be the result of enhanced rates of tubular fluid flow at the higher levels of sodium intake (10).

Urea clearance increased significantly with increasing sodium intake, suggesting that glomerular filtration rate increased in man with sodium loading. The phenomenon was observed by Kirkendall *et al.* (8) using inulin clearance.

Plasma renin activity and plasma aldosterone concentrations decreased progressively

with increasing sodium intake. Kirkendall *et al.* (8) noted a similar relationship between plasma renin activity and urinary aldosterone excretion. Conceivably, the suppression of the renin-angiotensin-aldosterone system coupled with an increase in glomerular filtration rate served to permit our subjects to excrete enormous sodium loads. The possible participation of other systems cannot be ascertained from these studies. The increase in arterial blood pressure observed in our patients may be attributed to an increase in cardiac output. Permission was not obtained in our study to measure right atrial pressure directly at each level of sodium intake; however, assuming that the value remained constant at 5 mm Hg, systemic vascular resistance decreased in our subjects from 1171 to 1092 dynes/sec/cm<sup>-5</sup>. It is likely that right atrial pressure increased during the study, which suggests that the actual decrease in systemic vascular resistance was greater than our estimate. These short term studies do not address the concept of whole body circulatory autoregulation (5). Long term studies at extremes of sodium intake would be necessary to determine whether or not an increase in systemic vascular resistance would eventually be provoked.

Guyton and colleagues (5) postulate that hypertensive disorders are characterized by quantitative and/or qualitative alterations in the kidney's ability to excrete sodium at a given blood pressure. Our results support Guyton's conceptual relationship as applied to normotensive individuals; however, we can make no comments about the relationships between the state of sodium balance and systemic blood pressure in hypertension. Appropriately modified protocols applied to subjects with various categorized forms of hypertension will be necessary to define the kidney's behavior under these conditions.

**Summary.** The relationship between blood pressure and the state of salt balance was evaluated at four levels of salt intake (10 mEq/day, 300 mEq/day, 800 mEq/day, and 1500 mEq/day) in eight normal men. Increasing salt intake resulted in progressive increases in weight, blood pressure, potassium excretion, and creatinine clearance, while plasma renin activity and plasma aldosterone concentration decreased. Cardiac output in-



creased with increasing salt intake, while calculated systemic vascular resistance decreased. The curve defining the relationship between salt excretion and blood pressure was derived. These results support the conceptual framework integrating blood pressure regulation through the final common pathway of renal salt excretion. Moreover, they underscore the importance of salt regulation in the pathogenesis of hypertension.

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## Red Cell Oxygen Affinity in Severe Hypertriglyceridemia<sup>1</sup> (40365)

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The observed relationship between hyperlipemia and angina by Kuo *et al.* led to his thesis that hypertriglyceridemia might impair both oxygen uptake from the lungs and oxygen delivery to the tissues (1). Subsequent studies on oxygenation in hyperlipemic subjects focused on the finding of arterial hypoxemia (2, 3), until Ditzel (4, 5) recently described a series of severely hyperlipemic patients with a markedly increased affinity of hemoglobin for oxygen (low  $P_{50}$ ). This abnormality was unusual in that red cell 2-3 diphosphoglycerate (DPG) levels were normal and the high oxygen affinity could be corrected by incubating the patient's red cells in normal plasma. The authors suggested that this abnormality would interfere with oxygen delivery to active muscle, providing an explanation for the observations of Kuo. There is no known mechanism to explain a reversible reduction in standard  $P_{50}$  (at  $P_{50} = 37^\circ$ , pH = 7.40,  $PCO_2 = 40$  torr) by as much as 6-10 torr in the presence of normal DPG levels; the present study was designed to further investigate the relationship between hypertriglyceridemia and in vivo red cell oxygen affinity.

**Methods and materials.** Blood was obtained from seven subjects with hypertriglyceridemia due to a variety of causes (Table I) at a time when their serum was lipemic (TG =  $2213 \pm 2213$  mg/dl,  $\bar{X} \pm SD$ ) and from two normal subjects with triglyceride levels less than 100 mg/dl. Hemoglobin oxygen affinity was measured for all subjects by the tonometer technique (6) using a rotating flask tonometer (7) and Radiometer blood gas electrodes. A blood-gas  $O_2$  correction factor (tonometer gas  $PO_2$ /tonometer blood  $PO_2$ ) calculated from normal blood was measured

daily and applied to all  $P_{50}$  calculations. Results were expressed as  $P_{50}$  standardized to pH of 7.40,  $PCO_2$  of 40 torr, and temperature of  $37.0^\circ$  by the standard correction factors for human blood (8). Patients 5, 6 and 7 (Table I) also had  $P_{50}$  measured by the dissociation curve apparatus (DCA) of Duveleroy *et al.* (9). Both techniques are performed routinely in our laboratory, with standard deviation of 0.5 torr by the mixing technique and 0.4 torr with the DCA apparatus from eleven aliquots of the same sample of human blood. DPG concentrations, expressed as  $\mu$ g/ml of packed red cells, were measured by the method of Dettler *et al.* (10). The blood-gas oxygen correction factors for normal and hypertriglyceridemic blood were compared by tonometering samples for 30 min with 21%, 7%, or 4.5%  $O_2$  prior to blood  $PO_2$  measurement, using the flask tonometer and blood gas electrodes described.

Incubation studies comparing normal blood (plasma TG=72 mg/dl) with hypertriglyceridemic blood (plasma TG=1625 mg/dl) were performed after the separated red cells were washed and spun three times in buffered normal saline. Three serial two-fold saline dilutions of both the normal and lipemic plasma were prepared. One volume of packed normal red cells was added to 1 vol of each normal plasma sample, and 1 vol of packed lipemic red cells was added to 1 vol of each lipemic plasma sample. The mixed samples were tonometered for 30 min with room air prior to blood gas measurements. Spectrophotometric measurements of hemoglobin concentration and oxygen saturation were made on a model 182 Cooximeter (Instrumentation Laboratories) calibrated with normal human blood. Oxygen content of the tonometered resuspended mixtures was measured directly with a Lex- $O_2$ -Con (Lexington) oxygen analyzer.

**Results.** In this group of severely hypertriglyceridemic subjects, the mean  $P_{50}$  measured

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TABLE I. RBC OXYGEN AFFINITY IN LIPEMIA.

Subject	Cause of hypertriglyceridemia	Plasma triglyceride mg/dl	P <sub>50</sub> STD (torr) mixing technique	P <sub>50</sub> STD (torr) duvelleroy apparatus	DPG $\mu$ g/ml packed RBC
1	Familial and untreated diabetes	6600	28.6	—	4.4
2	Primary lipoprotein lipase deficiency	6048	27.3	—	4.3
3	Broad beta disease and untreated diabetes	2190	27.0	—	6.6
4	Primary lipoprotein lipase deficiency	970	29.0	—	4.3
5	Primary lipoprotein lipase deficiency	4560	27.7	19.1	3.6
6	Familial and untreated diabetes	2475	26.2	20.8	4.9
7	Familial and estrogen therapy	1764	28.2	22.0	5.2
		3515 $\pm$ 2213	27.7 $\pm$ 1.0	20.6 $\pm$ 1.5	4.8 $\pm$ 1.0

by the mixing technique was not different from normal (Table I). A substantial discrepancy was observed in  $P_{50}$  values measured concurrently by the DCA technique in subjects 5-7. The mean DPG concentration was normal, although there was considerable scatter in this value which did not correlate with the measured  $P_{50}$ . This variability may be related to the need to express DPG values per unit of packed red cells rather than per gram hemoglobin, since lipemic plasma causes an artifact in the spectrophotometric measurement of hemoglobin concentration (11). Although the plasma from the DCA chamber after a run showed evidence of hemolysis, in no case did the hematocrit fall by more than 2%. It thus appears that there was insufficient free hemoglobin to account for the decrease in  $P_{50}$  by the DCA measurement.

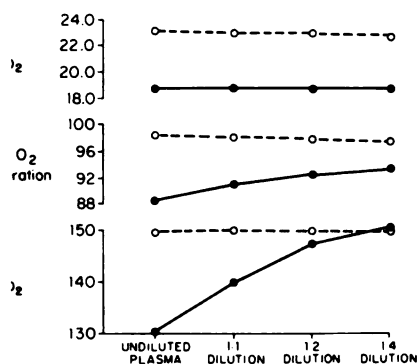
The blood-gas oxygen correction factors calculated at three PO<sub>2</sub> values on the tonometered lipemic blood (Table II) show that the correction value in the PO<sub>2</sub> range of the measured  $P_{50}$  is only about 5% greater than that for normal blood. Although these differences are relatively small, a second experiment demonstrated that this error is magnified considerably when lipemic blood remains in contact with the PO<sub>2</sub> electrode for fifteen minutes. In this study either lipemic blood or normal blood was held on the electrode for 15 min and then a sample of normal blood tonometered in 5% CO<sub>2</sub> and 20% O<sub>2</sub> was drawn into the chamber and the measured PO<sub>2</sub> was recorded. The baseline correction factor was 1.07, the correction factor

TABLE II. PO<sub>2</sub> CORRECTION FACTORS FOR NORMAL AND LIPEMIC BLOOD.

Tonometer PO <sub>2</sub> torr	Normal blood correction	Lipemic blood correction
148	1.07	1.21
50	1.03	1.08
32	1.03	1.08

after 15 min of incubation with normal blood was 1.31, and the factor with lipemic blood was 2.00. Thus prolonged contact between lipemic blood and the PO<sub>2</sub> electrode such as occurs during a DCA measurement can result in a measurement error of sufficient magnitude to account for the  $P_{50}$  differences observed between the mixing technique and the DCA.

The final experiment was performed to expand on the previous observation that the low (DCA)  $P_{50}$  of lipemic blood could be corrected by incubation with normal plasma (5). The washed red cells of normal and lipemic blood were incubated in a tonometer with serial saline dilutions of the normal and lipemic plasma, respectively. Measurement of O<sub>2</sub> content, O<sub>2</sub> saturation and PO<sub>2</sub> for each dilution of the blood samples (Fig. 1) shows that while the O<sub>2</sub> content of the lipemic blood remains unchanged with saline dilution of the plasma, there was a progressive increase in both measured O<sub>2</sub> saturation and measured PO<sub>2</sub>. This discrepancy was not seen with serial dilution of normal plasma. (The blood from the normal subject had a higher hematocrit, accounting for the higher measured oxygen content at all dilutions.)



1. Effects of serial dilution of lipemic (—•—) and nonlipidemic (---○---) plasma with saline on dissolved oxygen content (CO<sub>2</sub>, by Lex-O<sub>2</sub>-Con), saturation (by Co-oximeter) and partial pressure (by PO<sub>2</sub> electrode).

**Discussion.** The affinity of hemoglobin for oxygen in blood from severely hypertriglyceridemic patients is normal, and our findings indicate that the previous reports of low  $P_{50}$  measured with the DCA apparatus in these patients are incorrect because of the inaccuracies associated with measurement of  $P_{50}$  in lipemic plasma. Lipemic plasma interferes with measurement by the standard methods of both PO<sub>2</sub> and hemoglobin oxygen tension. The DCA apparatus is particularly susceptible to this PO<sub>2</sub> measurement artifact, as the inscription of a full dissociation curve requires that the oxygen electrode be in contact with the blood for up to 15 min. The problem with the measurement of PO<sub>2</sub> in hypertriglyceridemic blood was noted by Sundstrom *et al.* (12) in a study of patients receiving infusions of a triglyceride emulsion (lipid), which has physiological properties similar to chylomicrons *in vivo*. They also noted that there was an artifactual decrease in spectrophotometrically measured oxygen saturation, although the relation of these effects to triglyceride levels was not studied. Blood with added Intralipid gives a falsely elevated hemoglobin concentration by spectrophotometric measurement (11), thus the per cent oxygen saturation calculated from this measurement is underestimated. Neither we nor others (5, 12) could demonstrate any effect on the measured PO<sub>2</sub> of lipid added to tonometered samples. The reason for this discrepancy may be related to uncharacterized physical chemical

differences between chylomicrons and Intralipid particles.

Ditzel (5) had suggested that the increase in oxygen affinity could be related to defects in the red cell membrane. Clinical reports have suggested that red cells from some severely lipemic patients are susceptible to hemolysis ("Zieve's Syndrome"), but in fact *in vivo* hemolysis has not been demonstrable in these patients (13). Lipemic blood is susceptible to *in vitro* hemolysis however (13), and this was apparent from the appearance of the lipemic plasma after exposure to the magnetic stirrer in the DCA apparatus in the present study. When the  $P_{50}$  was measured in lipemic blood by the mixing technique following 15 min of stirring in the DCA apparatus, the value was 2–3 torr less than the initial mixing technique value, suggesting that *in vitro* erythrocyte damage could also decrease the  $P_{50}$ . Nevertheless the DCA induced red cell trauma was not sufficient to decrease the hematocrit by more than 2%, and presumably the major portion of the low  $P_{50}$  artifact was related to the oxygen electrode problems.

These results coupled with the findings of Sundstrom *et al.* (12) suggest that previous reports of low arterial PO<sub>2</sub> values or low arterial O<sub>2</sub> saturation related to high triglyceride concentrations should be reevaluated with careful attention to the blood-gas correction factors for lipemic plasma. At present any abnormality of either arterial oxygenation or tissue oxygen delivery remains unestablished, and other factors need be sought to explain the clinical findings of lipemia associated angina or decreased exercise tolerance (14).

**Summary.** The clinical manifestations of impaired oxygen transport in severely hypertriglyceridemic patients have been attributed to a reversible increase in red cell oxygen affinity (low  $P_{50}$ ) in recent studies. In seven patients with comparably lipemic plasma (triglyceride levels 970–6600 mg/dl) the mean standard  $P_{50}$  measured by the mixing technique was normal. However when measurements were repeated on three of the samples using the Duvelleroy dissociation curve apparatus, the measured  $P_{50}$  was decreased by 5–9 torr. This difference was secondary to a time dependent interference of the lipemic plasma with the blood O<sub>2</sub> electrode, increas-

ing the blood-gas O<sub>2</sub> correction factor. The red cell oxygen affinity of subjects with severe hypertriglyceridemia is normal and other explanations need be sought for the clinical observations suggesting a decrease in tissue oxygen delivery.

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## Maintenance of Pregnancy in the Rat in the Absence of LH<sup>1</sup> (40366)

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The significant role of LH in pregnancy in rat was revealed by studies utilizing spenisera to LH (LHAS) (1, 2). Madhwa and Moudgal (2) demonstrated that S was capable of delaying implantation en before day 4. This study also showed LHAS terminated pregnancy when given ys 8 through 11, further identifying the for LH during this period (3-5). A xquent study (6) also confirmed the LH rement and showed further that inhibi- of prolactin secretion by ergocornine- d resorption of the conceptuses when ed on days 6 and 7 but not if given after '. Their study also suggested that a pla- l luteotropin could substitute for pitui- prolactin after day 7 and this has been ssfully proven (7). Thus, it appears that are changing requirements for LH and ctin during pregnancy and this may also e of the steroids.

tal resorption as a consequence of LHAS nistration has been correlated with re- on of progesterone secretion in many al models (8, 9). However, fetal resorp- n response to LHAS may also be related duced estrogen secretion during days 8 gh 11 (10).

at blastocysts survive in utero beyond the l time of implantation if two conditions et. First, that sufficient progesterone is ble to insure their survival and second, there is no estrogen available to cause antation. In this state implantation can itiated by the provision of small amounts rogen.

e two conditions causing delay of im- ation can be met by autografting the or pituitary beneath the kidney capsule e implantation. This experimental

model has adequate prolactin from the graft to support sufficient progesterone secretion to insure blastocyst viability. Conversely, there is insufficient gonadotropin secretion to induce estrogen secretion to initiate implanta- tion. Thus, the blastocysts remain unim- planted until estrogen is administered.

The day estrogen administration begins in rats experiencing delay of implantation be- comes equivalent to day 4 of normal pre- gnancy. In the pituitary autografted model estrogen must be given for 9 consecutive days, equivalent to days 4 through 12 of normal pregnancy. Estrogen induces implantation and insures fetal survival beyond day 16 equivalent (7). This experimental model provides the opportunity to test if specific LHAS is capable of acting at any site other than by neutralizing maternal pituitary LH.

*Methods and materials.* Sprague-Dawley rats used in these experiments were housed in a temperature, humidity and light (14L-10D) controlled room. They were provided food and water *ad libitum*. Females were caged with experienced males and day 1 of pregnancy was the day sperm were found in the vaginal lavage. Parapharyngeal hypophy- sectomies were performed on day 2 and were later confirmed complete at autopsy. The an- terior pituitary gland of each female was autografted (APtr) beneath the kidney cap- sule (7). Successive laparotomies were per- formed on days 8, 12, 16, and 20. No fetal sites were found on day 8 because blastocyst implantation was delayed due to the absence of estrogen. Estradiol-17 $\beta$  (E-17 $\beta$ ) (Sigma Chemical Co.) was administered in sesame oil (0.1  $\mu$ g/day, days 8 through 12) by sub- cutaneous injection. This E-17 $\beta$  induced im- plantation and sites were visible on day 12. Thus, because of the 4 day delay in implan- tation days 8, 12, 16 and 20 became equiva- lent to day 4, 8, 12 and 16 of a normal pregnancy.

Antiserum to LH (LHAS) was prepared by

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immunizing rabbits with Papkoff ovine LH in Freund's complete adjuvant. The antiserum was rendered "monospecific" to LH by absorption with dilute normal sheep serum. The specificity of this material was assessed using the Ouchterlony diffusion technique. This test determined there was no cross reactivity with dilute normal sheep serum, ovine liver extract, NIH-FSH or NIH prolactin. However, a single clear precipitin band developed relative to Papkoff-LH with no spurs indicating no cross reactivity with the tissue preparations or the other pituitary hormones (2). The LH neutralizing capacity of this antiserum in our animals was assessed by determining the amount required to induce abortion. When given as one subcutaneous injection on day 8 of pregnancy, 0.7 ml of the antiserum induced total resorption. The antiserum dose used in the present experiments was one ml or 1.4 times the dose producing 100% resorption.

**Results.** LHAS given the day before the implantation-inducing E-17 $\beta$  treatment failed to delay nidation or to cause later resorption of the fetal sites (Table I). Administration of single doses of LHAS on days equivalent to days 7, 8, 9, 10 or 11 also failed to cause fetal resorption. Further, individual rats given aborting doses of LHAS on days 9 and 10 equivalent, did not show signs of fetal resorption.

**Discussion.** Previous experiments using hypophysectomized and pituitary autografted rats have been subject to the criticism that some small amount of LH may have been available for continuing luteal function from basophilic cells on remnants of the pituitary stalk or from cells of the grafted pituitary. The current experiments were designed to neutralize any LH with LHAS in the pregnant rat, which was hypophysectomized and pituitary autografted and treated with adequate amounts of estrogen to support pregnancy. Neither blastocyst survival nor subsequent implantation was influenced by the administration of LHAS. Most importantly, LHAS did not cause fetal resorption when given on days equivalent to days 8 through 11 of pregnancy. Thus, the corpus luteum can function at a physiological level in the absence of LH.

Blastocyst implantation and maintenance of pregnancy in the model used was dependent upon two factors, the continuing secretion of progesterone from the corpus luteum maintained by prolactin from the autografted pituitary and the provision of exogenous estrogen (estradiol-17 $\beta$ , 0.1  $\mu$ g/day) (7). This augers toward the concept that estrogen secretion induced by LH (11, 12) may be acting on the uterus in concert with progesterone to accommodate the rapidly expanding fetuses. Estradiol may also act upon the corpus lu-

TABLE I. EFFECT OF LH ANTISERUM (LHAS) ON THE BLASTOCYTES OR IMPLANTATION SITES OF RATS BEARING PITUITARY AUTOGRAPHS.<sup>a</sup>

Ref Group	LHAS RD <sup>b</sup>	Treatment		Observations (RD)					
		Estradiol 0.1 $\mu$ g/day		8		12		16	
		RD	CD <sup>c</sup>	♀S/♀T <sup>d</sup>	$\bar{X}_s$ <sup>e</sup>	♀S/♀T	$\bar{X}_s$	♀S/♀T	$\bar{X}_s$
21	3	4-12	8-16	4/4	10.0 $\pm$ 1.0	3/4	10.3 $\pm$ 1.3	3/4	9.7 $\pm$ 1.2
19 and 23	7	4-12	8-16	1/1	13	1/1	13	1/1	13
	8	4-12	8-16	6/6	11.7 $\pm$ 1.8	5/6	10.6 $\pm$ 1.5	5/6	9.2 $\pm$ 1.5
	9	4-12	8-16	5/5	12.8 $\pm$ 1.2	4/5	10.8 $\pm$ 2.2	4/5	7.0 $\pm$ 1.4
	10	4-12	8-16	3/3	9.7 $\pm$ 2.7	3/3	9.7 $\pm$ 2.7	3/3	8.0 $\pm$ 2.5
	11	4-12	8-16	1/1	12	1/1	12	1/1	10
24	9, 10	4-12	8-16	3/3	10.3 $\pm$ 0.3	3/3	10.0 $\pm$ 0.6	2/2 <sup>f</sup>	9.0 $\pm$ 0.0

<sup>a</sup> Anterior pituitaries grafted on day 2, the day after sperm were seen in the vaginal lavage.

<sup>b</sup> RD—Relative day of normal pregnancy.

<sup>c</sup> CD—Chronological day after day 1.

<sup>d</sup> ♀S/♀T—females with sites/total females observed.

<sup>e</sup>  $\bar{X}_s$ —mean number of sites  $\pm$  SE in females with sites.

<sup>f</sup> One animal died.

to promote progesterone secretion in the presence of anterior pituitary or placental LH.

It is interesting to note that Madhwa Raj and Moudgal (2) were unable to thwart the effect of LHAS by the administration of estrogen.

Yet, in the model under consideration, which was reported in a previous study, estrogen administration was a *sine qua non* for the maintenance of the fetus from day 8 to day 11. Additionally, estrogen alone was not sufficient to maintain pregnancy following hysterectomy accomplished during this early phase of pregnancy. The reason for the failure or success of fetal maintenance during pregnancy awaits insight derived from further studies.

Although LHAS depressed progesterone secretion and caused resorption in the intact pregnant rat, these seemingly associated effects may not be cause and effect. It is possible that estrogen secretion was also repressed by the administration of LHAS. The results of studies of the capacity of LHAS to maintain pregnancy seem to amply illustrate that LHAS regulates both progesterone and estrogen secretion.

The conclusion of this study demonstrated that pregnancy does not require the presence of LH between days 8 and 11. Previous experiments (7) showed there was an estrogen requirement for pregnancy maintenance in the rat. The current study implies that there is a role for LH in pregnancy to maintain progesterone secretion. This work also confirms the concept, long accepted but unproven, that LHAS acts specifically against pituitary LH-like material and denies the possibility that LHAS acts upon the unimplanted blastocyst, the implanting blastocyst, or the development of the placenta.

**Materials and Methods.** Pregnant rats were hypophysectomized and pituitary autografted on day 2, 3, or 4 after sperm were observed in the vaginal lavage. Estradiol-17 $\beta$  (E-17- $\beta$ ) was administered (0.1  $\mu$ g/day) on days 8 through 16 to

induce implantation and maintain pregnancy. This protocol resulted in a 4 day delay of implantation, and day 8 becomes equivalent to day 4 of normal pregnancy. A single dose of LHAS (equivalent to 1.4 times the dose necessary to cause abortion on day 8 in the normal pregnant rat) failed to prevent implantation when administered on day 7 or cause fetal resorption when administered on days 11, 12, 13, 14 or 15 (equivalent to days 4, and 7 through 11). LHAS given on the two successive days 13 and 14 (days 9 and 10 equivalent) was also without effect. These results suggest that LHAS causes abortion in the rat by acting on pituitary LH-like material and not on the ovary, developing fetus or placenta.

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# ***In Vitro* Analysis of the Participation of Oxytocin and Vasopressin in the Gonadotropin Releasing Hormone-Induced Release of LH<sup>1, 2</sup> (40367)**

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Both anatomical (1) and physiological (2) evidence suggest that the neurohypophyseal hormones may be involved in the control of the anterior pituitary gland. Cells of the anterior pituitary are exposed to concentrations of vasopressin (AVP) and oxytocin (OT) that are hundreds of times greater than those found in the peripheral circulation (3, 4). One aspect of the question of anterior-posterior pituitary interactions concerns the influence of neurohypophyseal hormones on the secretion of gonadotropins. In fact, pituitary stores of the neurohypophyseal hormones have been found to vary according to the estrous cycle of the rat, the highest concentrations being measured at estrus and proestrus followed by a marked depletion during diestrus (5). In humans, peripheral levels of AVP exhibit a rhythmic pattern during the menstrual cycle (6). An analysis of the menstrual cycle of the monkey revealed a peak of estrogen-stimulated neurophysin, the carrier protein thought to be associated with OT, which coincided with the peaks for estrogen and LH prior to ovulation (4). The hypothesis that there may be some relationship between AVP and gonadotropin releasing hormone (GnRH) was first tested in 1964 by Sakiz and Guillemin (7) who used a bioassay for LH, the ovarian ascorbic acid depletion (OAAD) assay. A dose of synthetic AVP with no activity in the OAAD assay did not further enhance the test response when used in combination with LH. However, when the same dose of AVP was administered concomitantly with hypothalamic extracts, OAAD activity

was increased over controls. More recent *in vivo* studies in the rat also suggest an interaction between AVP and GnRH. When a tripeptide identical to the terminal amino acid sequence of AVP was injected into chlorpromazine-blocked proestrus rats, there was an increased number of ova shed 18 hours later in response to GnRH or LH (8). In another experiment (9), lysine vasopressin increased the ovulatory response in immature rats primed with pregnant mare serum gonadotropin. There is some indication, then, that the neurohypophyseal hormones may affect gonadotropin secretion via an interaction with GnRH. The purpose of the present experiments was to clarify the relationship between the neurohypophyseal hormones and GnRH using an *in vitro* approach.

**Materials and methods.** Anterior pituitary glands from male Wistar rats (170-350 g) were used for all perfusion experiments. Rats were decapitated immediately before each experiment and the pituitary gland was removed. The posterior pituitary was discarded and the anterior pituitary was hemisected. The halved anterior pituitaries (hemipituitaries) were kept at room temperature in fresh Krebs-Hensleit buffer containing 0.2% (w/v) glucose and oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub> until all the tissue had been collected. Each hemipituitary was then placed in a Tygon tubing chamber (length, 10 mm; inner diameter, 3 mm) which was narrowed at either end by the attachment of glass micropipet tips. Glass wool was inserted into the pipet tip leading to the collection tubes in order to prevent exit of cellular material from the chamber. All hemipituitaries were perfused with control buffer for two hours in a shaking water bath at 34° to obtain a steady baseline secretion of LH. The tissue was continuously perfused at a rate of 0.5 ml/min with Krebs-Hensleit buffer (plus 0.1% gelatin) that was oxygenated and warmed to 34° before com-

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in contact with the tissue. Effluent was collected at four minute intervals, kept ice-cold throughout the experiment, and stored at 0° until assayed. After the effluent had been collected for 20 minutes, the pituitaries were perfused with hormone-containing media. Administration of the hormone was accomplished by quickly transferring the syringe carrying the perfusion media to the perfusion chamber without altering the speed of the perfusion pump. Hormone treatments were: control (0.02, 0.2, 2, 20 or 200 mU/ml); 0.2 ng/ml GnRH plus 0.02, 0.2, 2, 20 or 200 mU/ml; 0.2 ng/ml GnRH plus OT (0.002, 0.02, 0.2, 2 or 20 U/ml); AVP alone (0.02, 0.2, 2, 20 or 200 U/ml); or OT alone (0.02, 0.2, 2 or 20 U/ml). In some experiments, 2 and 20 ng/ml AVP or 0.02 and 0.2 mU/ml OT were added to the media in the initial 20-min perfusion period as well as during GnRH treatment. The dose of GnRH (0.2 ng/ml) was shown in our previous experiments to cause submaximal release of LH and would, therefore, allow for any possible potentiating effects by AVP or OT to be easily detected. A fresh solution of GnRH was prepared on the day of each experiment. AVP and OT were stored at -40° in a stock solution of 400 U/ml dissolved in 0.3% acetic

acid. Synthetic GnRH was obtained from the Hormone Distribution Officer, NIDDK as Abbott lot 26-306AL. Synthetic GnRH (biological activity, 385 U/mg) and OT (biological activity, 425 U/mg) were obtained from Bachem, Inc. Pooled samples from experiments in which hemipituitaries were perfused with different doses of AVP were assayed for ACTH in the laboratory of W. Kendall to confirm biological activity of the hormone preparation. 200 mU AVP and samples from 3 hemipituitaries) showed a 100% increase in baseline ACTH release for the entire 20-min of exposure to hormone.

**Radioimmunoassay.** All LH radioimmunoassays were performed according to the method of Niswender *et al.* (10). Each four-minute effluent was assayed in 500  $\mu$ l duplicate. The sensitivity of the assay was 29.0 ng (n=25) using NIH-LHS18 as the LH standard. Coefficients of variation were 10% (n=25) for interassay and 8.2% (n=9) for intra-assay variation.

**Analysis of data.** In order to eliminate interassay and inter-animal variation, data collected from each hemipituitary were recorded as percent of baseline over time. The baseline was calculated as the mean value of the four samples taken in the 20 min before the hormone-administration period. For statistical analysis, a cumulative value of the percent of baseline data was determined for each hemipituitary in an experimental group. This value was essentially a measure of the area under a hypothetical curve which could be plotted with the values from each individual hemipituitary. The resulting cumulative value was then expressed in terms of percent of baseline attained by each gland per sample collection period. The mean of these final values was used as the standard of comparison between groups. Dunnett's test (11) was used to determine significant differences.

**Results. Experiment I.** In this experiment (Table I), the responsiveness of the luteotropin to AVP and OT was examined. LH was measured in effluent from rat hemipituitaries perfused with different concentrations of AVP or OT. All hemipituitaries were perfused with control buffer for the first 2 hr of each experiment resulting in a mean steady baseline of  $1.68 \pm 0.12$  (SE) ng LH/ml (n=114). Responses of the hemipituitaries were then followed for 44 min. For the first 20 min, the hemipituitaries in experimental groups were treated with AVP or OT-containing media. For the remaining 24 min all

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF AVP AND OT ON LH RELEASE IN PERFUSED RAT HEMIPIUITARIES.<sup>a</sup>

Treatments	Neurohypophyseal hormone concentrations (mU/ml)					
	0 <sup>b</sup>	0.02	0.2	2.0	20	200
AVP	(4) <sup>c</sup>	(4)	(4)	(3)	(3)	(3)
	91 <sup>d</sup>	127*	99	95	87	78
	$\pm 7^*$	$\pm 17$	$\pm 9$	$\pm 13$	$\pm 6$	$\pm 16$
OT	(4)	(4)	(4)	(3)	(3)	
	91	114*	119*	103	122*	
	$\pm 7$	$\pm 7$	$\pm 13$	$\pm 5$	$\pm 9$	

<sup>a</sup> Rat hemipituitaries were perfused first with buffer for a control period to determine baseline then with AVP or OT for 20 min followed by a 24-min washout period.

<sup>b</sup> Control, no hormone added.

<sup>c</sup> Number of hemipituitaries perfused.

<sup>d</sup> Percent of baseline per sample collection period.

\* Mean  $\pm$  SE.

\*  $P < 0.05$ .

groups were perfused with hormone-free perfusion media. After most concentrations of AVP, treated hemipituitaries did not differ from controls. However, after 0.02 mU/ml AVP, release of LH was significantly elevated ( $P < 0.05$ ) over the control value (Fig. 1). All concentrations of OT tested, except the 2 mU/ml concentration, were able to induce LH release (Fig. 1).

**Experiment II.** The purpose of this experiment (Table II) was to investigate the combined effects of neurohypophyseal hormones and GnRH on the release of LH. Tissue was perfused with either GnRH or GnRH plus different concentrations of AVP or OT for 20 min following the two hour perfusion with control buffer. Hemipituitaries stimulated with 0.2 ng/ml GnRH increased baseline LH release ( $P < 0.01$ ) over controls. Doses of AVP ranging in concentration from 0.02 to 200 mU/ml did not alter tissue response to GnRH. Only one concentration of OT, 0.02 mU/ml, potentiated ( $P < 0.05$ ) the LH response to GnRH while concentrations that were tenfold greater or smaller had no effect. A graph of the data (Fig. 2) shows that at this dose OT increased the magnitude and prolonged the release of LH from the hemipituitaries in response to 0.2 ng/ml GnRH.

**Experiment III.** This experiment was performed to determine if a greater release of LH could be induced by a longer exposure time to the neurohypophyseal hormones. Hemipituitaries were perfused with the hor-

mones according to the schedule described for experiments I and II, but in addition were treated with either AVP or OT in the 20-min period prior to the usual 20 min of GnRH stimulation. This modification altered the response seen after acute exposure to the hormones. The addition of either 2 or 20 mU/ml AVP prior to as well as during GnRH treatment resulted in a mean cumulative percent of baseline per collection period of  $233 \pm 40$  SE ( $n=4$ ) for the lower and  $294 \pm 26$  SE ( $n=4$ ) for the higher dose. These values represent a significant ( $P < 0.05$  and  $P < 0.01$ , respectively) and dose-related increase over GnRH control data as seen in Table II. In the same experiment, pre-exposure of the tissue to 0.02 mU/ml OT increased LH production in response to GnRH to  $283 \pm 39$  SE ( $n=4$ ;  $P < 0.01$ ). Hemipituitaries pretreated with 0.2 mU/ml OT responded to GnRH with a reading of  $168 \pm 29$  SE ( $n=4$ ) percent of baseline, a value similar to that for tissue perfused with GnRH alone.

**Discussion.** Results from *in vitro* studies in which release of anterior pituitary hormones is stimulated by various agents imply that multiple functional receptors may be present on the tropic hormone-producing cells. Our experiments demonstrate that neurohypophyseal hormones can influence LH secretion *in vitro*. Our finding that most concentrations of AVP, when used alone, are ineffective in releasing LH confirms studies by other investigators using *in vitro* pituitary incubation systems (12, 13). The LH releasing ability of 0.02 mU/ml AVP, however, is contradictory to previous reports in which only high concentrations of Pitressin released LH after long incubation periods (14). In an investigation of TSH release by neurohypophyseal hormones in incubated pituitaries, Krass *et al.* (15) also obtained a spike-like dose response curve for AVP when only one of four concentrations of AVP ( $8 \times 10^{-10}$  M or 0.3 mU/ml) caused significant release after 30 minutes. The failure of our single LH-releasing concentration of AVP to release LH in the presence of GnRH may have been due to the fact that the stimulatory effect of AVP was masked during the GnRH stimulation despite use of submaximal concentrations of the releasing hormone. Preexposure of the tissue to two previously ineffective higher concentra-

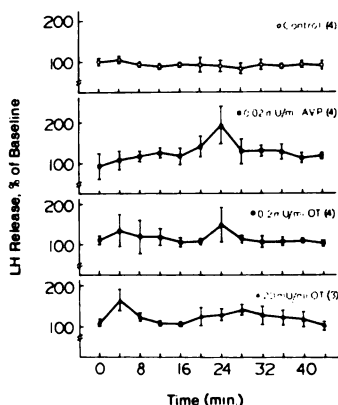


FIG. 1. LH release from perfused rat hemipituitaries which were exposed to buffer only (control) or to different concentrations of AVP alone (0.02 mU/ml) or OT alone (0.2 mU/ml, 20 mU/ml) from 0 to 20 min.

TABLE II. EFFECT OF DIFFERENT CONCENTRATIONS OF AVP AND OT ON GnRH-INDUCED RELEASE OF LH FROM PERFUSED RAT HEMIPITUITARIES.<sup>a</sup>

	Neurohypophyseal hormone concentrations (mU/ml)						
reatments	0 <sup>c</sup>	0.002	0.02	0.2	2.0	20	200
H <sup>b</sup> + AVP	(7) <sup>d</sup> 166*** ± 7 <sup>f</sup>		(6) 188 ± 23	(5) 193 ± 19	(8) 143 ± 15	(8) 180 ± 23	(4) 137 ± 13
H <sup>b</sup> + OT	(7) 166** ± 7	(5) 157 ± 7	(7) 243* ± 23	(7) 165 ± 11	(6) 151 ± 11	(5) 155 ± 18	

Rat hemipituitaries were perfused first with buffer for a control period to determine baseline, then for 20 min GnRH alone or GnRH plus either AVP or OT followed by a 24-min washout period.

0.2 ng/ml.

Treated with GnRH alone, 0.2 ng/ml.

Number of hemipituitaries perfused.

Percent of baseline per sample collection period.

Mean ± SE \**P* < 0.05. \*\**P* < 0.01 as compared to control in Table I.

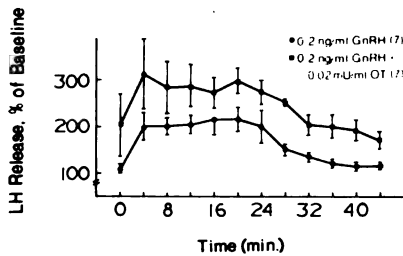


FIG. 2. LH release from perfused rat hemipituitaries were exposed to 0.2 ng/ml GnRH from 0 to 20 or to 0.02 mU/ml OT plus the same dose of GnRH 0 to 20 min.

s of AVP resulted in a significant enhancement of the subsequent GnRH-induced release of LH. This suggests that AVP may be responsible for the activation of some component of the stimulus-secretion coupling mechanism required for the release of LH from the luteotropin in response to GnRH.

In our *in vitro* system, OT alone was able to raise baseline LH production at most of the concentrations tested. The one ineffective dose, 2 mU/ml, is a concentration which may borderline between a physiological and a pharmacological concentration. Possibly, the response curve for OT is a plateau which is reached after 0.2 mU/ml. Krass *et al.* (15) reported a similar pattern for OT stimulation of LH release when three different concentrations of OT elicited equal responses while lower or higher doses were ineffective. The stimulation of LH release by 20 mU/ml OT may represent a nonspecific response. In a previous investigation, Wilks and Hansel (16), testing a wide range of concentrations, concluded that OT had no LH-releasing ability.

However, in their system, degradation of LH would have prevented the detection of the slight increase in LH seen under the conditions of our experiment. Only one concentration of OT in combination with GnRH was effective in releasing LH above control GnRH-stimulated values. When the tissue was preexposed to OT, this same concentration was still effective while a concentration tenfold higher was not, indicating that a precise ratio between GnRH and OT must be attained for a potentiating effect to take place. Although the GnRH-potentiating effect of OT is highly concentration dependent it is less so, in our pre-exposure experiments, for AVP. Preexposure of the anterior pituitary tissue appears to be necessary for the enhancement of the GnRH-induced release of LH by AVP whereas this pretreatment is not necessary in the case of OT. OT acts in concert with GnRH while AVP must act prior to GnRH, preparing the luteotropin for the stimulating effects of the releasing hormone.

It has been proposed that the modification of enzymatic degradation of peptide hormones at receptor sites could be one possible mechanism for the regulation of their actions (16). The fact that OT and GnRH are inactivated by the same peptidase has led to the hypothesis that elevations of OT in hypothalamic and portal blood during proestrus may enable GnRH stores to increase sufficiently to initiate the LH surge (17). This may be one of the mechanisms whereby OT enhances and/or prolongs the effect of GnRH on LH release in our pituitary perfusion system.

It is evident from the present experiments

that AVP and OT augment the GnRH-induced release of LH by the pituitary *in vitro*. The purpose of these and other peptide-peptide interactions might be the attainment of finer levels of control of secretion which would not be possible through only one hormone. The ability of AVP and OT to act alone leaves open the possibility of a completely independent mechanism although this might reflect an interaction with endogenously produced GnRH already occupying the receptor.

The results of the present studies show that AVP and OT can affect the release of LH and the responsiveness of the luteotrop to GnRH. Although evidence for participation of AVP and OT in the reproductive cycle is still not conclusive, the relationship between the neurohypophyseal hormones and GnRH warrants further investigation.

**Summary.** The pituitary perfusion technique was used to investigate the possible interaction between the neurohypophyseal hormones and gonadotropin releasing hormone (GnRH). The perfusion of rat anterior pituitaries with 0.2 mU/ml arginine vasopressin (AVP) resulted in a significant ( $P < 0.05$ ) increase in LH release over baseline, while higher doses had no effect. When combined with GnRH, this and higher concentrations of AVP did not alter the GnRH-induced release of LH. Three concentrations of oxytocin (OT): 0.02, 0.2 and 20 mU/ml, increased baseline secretion of LH ( $P < 0.05$ ) while 2 mU/ml OT did not. When added to GnRH-containing perfusion media, 0.02 mU/ml OT caused significant ( $P < 0.05$ ) enhancement and prolongation of the LH response to GnRH. All higher concentrations of OT and one concentration that was tenfold lower, did not exhibit potentiating effects. When the pituitary tissue was pretreated with AVP or OT prior to stimulation with GnRH, only the same concentration of OT (0.02 mU/ml) was effective ( $P < 0.01$ ) while two concentrations of AVP (2 and 20 mU/ml) which had been ineffective previously, then enhanced the LH release due to GnRH ( $P$

$< 0.05$  and  $P < 0.01$ , respectively). It is proposed that the data from these experiments support the hypothesis that AVP and OT may have a role in reproduction via an interaction with GnRH at the level of the anterior pituitary.

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## Oxygen Consumption in the Spontaneously Hypertensive Rat (40368)

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The spontaneously hypertensive rat (SH) has been widely utilized as an animal model for the study of physiological functions as they pertain to essential hypertension in man. In a recent report, Wright *et al.* (1) demonstrated a marked decrease in the responsiveness to thermal stress in the SH rat which they, in part, attributed to deficient water mobilization for evaporative cooling. The possibility remained that changes in metabolic heat production, cardiovascular adjustments to heating or other factors which might accompany the development of hypertension could influence thermoregulation of hypertensive animals in hot environments.

The work of Kloetzel *et al.* (2) demonstrating a high percentage of hypertensive individuals in industrial jobs requiring heat exposure has emphasized the need for studies to define stress responses in diseases such as hypertension in which the early symptoms may go undetected or the use of maintenance therapy combined with peer group pressure may encourage normal work and recreational activities. The limits which are described for physical exertion and exposure to environmental factors are nearly always based on data obtained from young, healthy individuals, and it may be reasonable to question their applicability to individuals exhibiting chronic alterations in physiological functions. In the present study, oxygen consumption and rectal temperatures of SH and normotensive rats were determined during exposure to a range of environmental temperatures designed to induce mild cold or heat stress. In an attempt to determine the nature of the observed differences, the effects of beta adrenergic blockade on O<sub>2</sub> uptake in cool and warm environments was examined.

**Materials and methods.** Male SH rats (blood pressure =  $163 \pm 7$  mm Hg and weight,  $341 \pm 7$  g) of the Okamoto-Aoki

strain (3) and parent strain Wistar-Kyoto (WKY) normotensive (bp =  $112 \pm 5$  mm Hg and weight,  $447 \pm 16$  g) rats were examined at 15–20 weeks of age. Animals were housed at  $24 \pm 1^\circ$  and 50% relative humidity with a 14 hr light–10-hr dark photoperiod. Water and Purina rat chow were available *ad libitum*.

Oxygen utilization was determined using the open flow system of Ben-Porat *et al.* (4). The animals were studied in 3.0 liter metabolic chambers submerged in a 170 liter waterbath held at  $\pm 0.1^\circ$  of the desired temperature. Room air was passed through the chamber at approximately 0.6 liter/min and the ambient temperature (Ta) in the chamber monitored with thermometers inserted into the chamber space. Water was absorbed on dryrite which had been incorporated into the inflow and outflow lines and in the chamber floor. Carbon dioxide absorbant (NaOH) was placed in the chamber floor and outflow lines. Air samples of about 1.0 liter were collected in Saran plastic bags at 30-min intervals over a 3.0-hr period for determination of O<sub>2</sub> content with the Beckman E-2 O<sub>2</sub> analyzer. In order to avoid variations in the data associated with the early adjustment of the animal to the chamber environment, only the last three values were averaged and compared. Pairs of SH and WKY animals were placed in the preheated chambers at either 0900 or 1300 hours (Eastern Standard Time) for exposure to a total of 5 ambient temperatures ranging from  $21^\circ$  to  $32^\circ$ . Animals were exposed to a single environmental temperature at each experiment with experiments spaced at 7-day intervals over a 5-week period. In order to minimize acclimatory effects and error related to circadian variations in data, exposure scheduling was randomized as to the sequence of exposure temperatures and was arranged in such a fashion that equal

numbers of animals in each group were utilized in morning and afternoon determinations. Body temperatures were determined before and at the end of each exposure with YSI thermister probes inserted 5–7 cm into the rectum. Oxygen consumption was calculated per metabolic body weight, ml O<sub>2</sub>/mm/kg<sup>-0.74</sup> (5).

Following the determinations in untreated animals, the effect of beta adrenergic blockage on the rectal temperature and O<sub>2</sub> utilization was examined at Ta 21° and 30°. Animals were administered propranolol (Inderal, Ayerst Laboratories; 3.0 mg/kg) by ip injection at 12 hr and immediately prior to placement in the metabolic chamber. Preliminary experiments with animals not used in this study indicated that this dose regimen resulted in an average 18% (74 bpm) drop in heart rate at 2 hr after the second injection.

Two weeks after the termination of the O<sub>2</sub> uptake studies the animals were anesthetized with Secobarbital sodium (Lilly, 0.06 ml/100 g) and blood samples were obtained by heart puncture for TSH, T<sub>3</sub> and T<sub>4</sub> determination by radioimmunoassay (6) of the serum. Animals were then sacrificed by cervical dislocation and the thyroid gland removed into 10% formalin for histological examination.

**Results.** The oxygen consumption of SH animals as compared to the WKY group was elevated at each temperature examined although the differences were statistically significant ( $P < 0.05$ ) only at temperatures above 25° (Fig. 1). Propranolol had no effect on the O<sub>2</sub> uptake of either the SHR and WKY groups at 21° but resulted in 14% and 12% decreases ( $P < .001$ ) in the normotensive and SH rats, respectively, at 30°. Propranolol did not, however, ameliorate SHR-WKY differences in O<sub>2</sub> consumption at 30°.

The SH rat rectal temperatures obtained at the end of the 3-hr exposure showed a slight (0.4–0.6°) elevation above control values at ambient temperatures above 25° (Fig. 1). The rectal temperature of the SH rats receiving propranolol was increased above the values recorded for untreated SH animals whereas no change was observed for the WKY group at Ta 21°. No effect on rectal temperature was noted in either group following propranolol administration at Ta 30°.

Histological examination of thyroid tissue

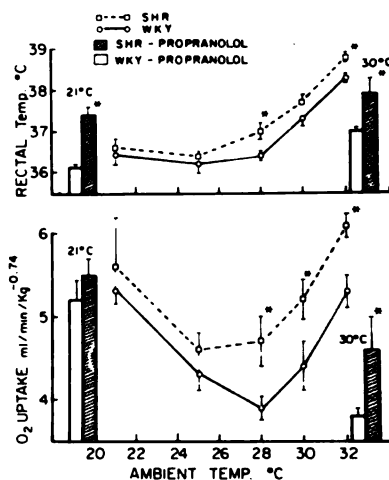


FIG. 1. Oxygen utilization and post exposure rectal temperatures of normotensive and spontaneously hypertensive rats at different ambient temperatures. An asterisk indicates significant differences from control,  $P < 0.05$  or greater. Data are presented as the mean  $\pm$  SE of 10 animals.

revealed no apparent differences between the WKY and SHR groups. In contrast, the serum T<sub>3</sub> levels were elevated ( $P < .005$ ) while T<sub>4</sub> concentrations tended to be lower in the hypertensive animals (Table I). Serum TSH levels were markedly elevated in SH rats, showing a more than 4-fold increase above those recorded for the normotensive controls.

**Discussion.** A comparison of oxygen utilization of SH and normotensive rats indicates an enhanced metabolic response (15–20%) to higher temperature environments in SH rats (Fig. 1). The corresponding increase in end exposure rectal temperature (0.4–0.6°) in the warm environments suggests that this phenomenon is related to the relative inability of the hypertensive animal to prevent body temperature elevation as compared to normotensive controls. It is not clear, however, as to whether the increase in O<sub>2</sub> utilization represents a major contributing factor to the elevation in rectal temperature observed in the SH rat or a manifestation of the increase in body temperature occurring as a result of defective thermoregulatory function in other heat loss effector systems. Elevation in O<sub>2</sub> uptake seen in the SH rat may result from the direct effect of the higher body temperature on metabolic rate or on the energy requirement for increased respiratory, behavioral

TABLE I. THE SERUM T<sub>3</sub>, T<sub>4</sub> AND TSH VALUES  
OBTAINED FOR NORMOTENSIVE AND HYPERTENSIVE  
RATS.

Weight (g)	T <sub>3</sub> (ng%)	T <sub>4</sub> (ng%)	TSH (ng%)
477 ± 16	43.1 ± 2.0	4.6 ± 0.2	19.3 ± 3.0
341 ± 7	54.2 ± 2.7 <sup>a</sup>	3.9 ± 0.2	81.8 ± 4.4 <sup>a</sup>

<sup>a</sup>Significant difference ( $P < 0.05$  or greater). Data are  
expressed as mean ± SE of 10 animals.

retory functions designed to increase  
s.

O<sub>2</sub> consumption measurements in  
and SH rats before and after admin-  
istration of the beta adrenergic blocking agent  
propranolol provide information concerning  
contribution of circulatory catechol-  
amines (7) and the activity of the sympathetic  
nervous system (8) to the difference in the  
metabolic rate between these groups. In both  
groups propranolol exerted a marked  
thermogenic effect at 30° ambient temper-  
ature whereas no effect was observed at 21°.  
The effect of administering propranolol  
depended upon the amount of sympathetic  
activity in the organs or tissues examined (9),  
results suggest that at 30° the animals of  
both experimental groups were in a similar  
state of thermal stress and increased sym-  
pathetic tone which was abolished by adminis-  
tration of propranolol, without affecting body  
temperature. As compared with normoten-  
sive WKY animals, it seems that spontane-  
ously hypertensive rats are hypersensitive to  
temperature stress. However, their ele-  
vated metabolic rate at high ambient temper-  
atures seems not to be related to an increased  
sympathetic tone. The slight effects of pro-  
pranolol on O<sub>2</sub> uptake in both groups at 21°  
as a relatively minor adrenergic influ-  
ence on the mechanism of rectal temperature  
regulation in SH rats which received propran-  
olol at 21° is not certain.

The findings of Kojima and co-workers  
indicate that thyroid function is reduced  
in the SH rat, suggesting that oxidation meta-  
bolism may also be expected to be reduced  
in these animals. On the other hand, it has  
repeatedly been shown that oxygen consump-  
tion is elevated in humans demonstrating es-  
sential hypertension (11, 13). Our observa-  
tions on circulating T<sub>3</sub> and T<sub>4</sub> levels and  
thyroid histology tend to suggest normal or

slightly increased thyroidal activity in SH  
rats. The remarkable elevation noted in hy-  
pertensive TSH levels is in agreement with  
the observations of Kojima *et al.* (7) and  
indicates a marked abnormality in thyroid  
function and a relatively refractory response  
to pituitary stimulation. The observation of  
elevated O<sub>2</sub> uptake in SH rats only at high  
ambient temperatures, however, suggests that  
factors unrelated to the pituitary-thyroid axis  
may be operant in the SH rat which would  
result in elevated metabolism, particularly in  
stress situations. For example, the hyper-  
responsiveness to adreno-sympathetic adrener-  
gic stimuli in hypertensive rats is well docu-  
mented (14, 15), and it is possible that stress  
induced activity in these systems might result  
in a disproportionate elevation in metabolic  
rates. In view of the complementary activity  
of thyroxine to the positive calorogenic effect  
of epinephrine (16), the nature of the influ-  
ence of the thyroid in SH animals should be  
investigated further.

**Summary.** Oxygen utilization was found to  
be elevated in SH rats as compared to control  
animals, and the difference was statistically  
significant at ambient temperatures above  
25°. Corresponding elevations in rectal tem-  
perature were noted, and it was concluded  
that the enhanced metabolic response was  
related to the relative inability of the hyper-  
tensive rat to prevent rectal temperature ele-  
vation during heat stress. It was not clear as  
to whether the increase in O<sub>2</sub> uptake was a  
causal factor or resulted from body temper-  
ature elevation due to defective heat loss by  
the SH rat. Propranolol induced a significant  
reduction in oxygen usage of both SHR and  
WKY groups at Ta 30° but not at 21° indi-  
cating an adrenergic influence on metabolic  
rate during acute heat stress which was absent  
during cooler exposures. Serum T<sub>4</sub> levels of  
SH rats were not significantly different from  
control values whereas T<sub>3</sub> levels were elevated  
(26%) indicating normal or slightly increased  
thyroid activity. In comparison, TSH levels  
were elevated fourfold indicating a markedly  
abnormal thyroid response to this hormone.

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# ation of Interferon-impaired Initiation Factor Activity *in Vitro* by cAMP and dsRNA (40369)

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have reported that treatment of mouse with homologous interferon results in a decrease in the ability of initiation factor 2 to bind with initiator tRNA (Met-tRNA<sub>i</sub>) and GTP (1). In the rabbit reticulocyte-protein synthesizing systems, polypeptide chain initiation is inhibited by a regulated translation inhibitor (HRI) in the presence of ATP. Inhibition is induced by a low concentration of double-stranded RNA (dsRNA), but prevented by addition of either excess eIF or cAMP (2, 3). Recent evidence showed that inhibition of polypeptide chain initiation by HRI involves the phosphorylation of eIF-2 by HRI associated protein kinase. In the interferon system it has also been reported that protein synthesis in cells from interferon-treated cells is delayed by low concentrations of dsRNA (10), which activates certain protein kinases, a decrease in the rate and production of a low molecular weight product of protein synthesis (11-17).

The present study was undertaken to study the effect of these nucleotides, high and low concentrations of dsRNA, and other control factors on the eIF-2 inhibitory mechanism induced by interferon in mouse L cells. The results provide evidence that substances which affect the activity of different protein kinases strongly influence the level of eIF-2 activity as shown in the rabbit reticulocyte system (4-9).

**Materials and Methods.** *Interferon treatment.* Exponentially growing mouse L cells (layer strain L929) were treated with interferon units/ml of mouse interferon activity: 10<sup>7</sup> units/mg) for 24 hr at the presence of 2% fetal calf serum. After treatment, the cells were harvested and washed with 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM KCl, 5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol.

*Met-tRNA<sub>i</sub> preparation.* Initiator tRNA (Met-tRNA<sub>i</sub>) was purified from rat liver us-

ing DEAE-cellulose and BD-cellulose, successively, and then charged with <sup>35</sup>S-methionine (22.3 Ci/mmol) using Met-tRNA synthetase purified from *Escherichia coli* as previously reported (1).

*Preparation of eIF-2.* The cells treated or untreated with interferon (about 4 × 10<sup>9</sup> cells) were homogenized in 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM KCl, 5 mM MgCl<sub>2</sub> and 2 mM dithiothreitol (DTT), then centrifuged for 20 min at 15,000 rpm. Ribosomes in this supernatant were further purified by 60% sucrose cushion gradient centrifugation as previously reported (1). The purified ribosomes were suspended in 10 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 2 mM DTT and 0.5 M KCl, and then gently stirred for 60 min at 4°. After centrifugation (45,000 rpm for 3 hr), solid ammonium sulfate (0.361 g/ml) was added to the supernatant. The precipitate was redissolved in 1.0 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 M KCl, 2 mM DTT and 5% glycerol. After dialysis against the same buffer, the crude eIF-2 preparation was stored at -20° and used within 1 month.

*Assay of eIF-2 activity.* Each 0.1 ml of the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 2 mM dithiothreitol, 50 pmoles of <sup>35</sup>S-Met-tRNA<sub>i</sub> (4,500 cpm/pmol) and amount of eIF-2 indicated in the text. The solution was mixed before and after the addition of eIF-2, then incubated for 10 min at 37° in the presence of 1 mM GTP. The ternary complex formation (<sup>35</sup>S-Met-tRNA<sub>i</sub>-eIF-2-GTP) was determined as previously reported (1).

*Chemicals.* <sup>35</sup>S-Methionine (22.3 Ci/mmol) was obtained from Schwarz/Mann, cAMP and derivatives of cAMP from P-L Biochemicals Inc., poly rI and poly rC from Miles Laboratories and crude mouse interferon from the Bionetics Corp.

*Results. Effect of cAMP and ATP on the eIF-2 activity from interferon-treated cells.*

Previously, we have reported that treatment of cells with interferon results in reduction of the activity of eIF-2 which interacts with Met-tRNA<sub>i</sub> and GTP to form a ternary complex (Met-tRNA<sub>i</sub>-eIF-2-GTP) (1). When mouse L cells were exposed to mouse interferon (300 units/ml) for 24 hr at 37°, the eIF-2 activity was reduced about 60–70% as compared with that from the untreated control (1). Experiments similar to those performed with rabbit reticulocyte lysates (2, 3) were done to test the effects of cAMP, cAMP derivatives and ATP on the activity of eIF-2 from interferon treated cells. Figure 1 shows that the activity of eIF-2 from interferon treated cells is increased by cAMP at concentrations between 10  $\mu$ M and 50  $\mu$ M. The optimum concentration of cAMP was 12  $\mu$ M and this dose increased eIF-2 activity 2.8 times. cGMP (data not shown) and derivatives of cAMP (Table I) did not substitute for cAMP. High concentrations of cAMP (higher than 1 mM) significantly inhibited the eIF-2 activity from both interferon treated and untreated cells (about 49%). ATP (1–3 mM) partly reversed the impaired eIF-2 activity from interferon treated cells. The increase of activity of eIF from interferon treated cells by 1 mM ATP was less than that obtained with cAMP. The effect of cAMP and ATP was further increased in the presence of Mg<sup>2+</sup> (1 mM) as

TABLE I. EFFECT OF cAMP AND ITS DERIVATIVE COMPOUNDS ON eIF-2 ACTIVITY FROM INTERFERON TREATED AND UNTREATED CELLS.\*

Compound tested	<sup>35</sup> S-Met-tRNA <sub>i</sub> in ternary complex eIF-2 from	
	Untreated cells	Interferon treated cells
	pmol	pmol
None	15.0	4.9
Adenosine 3':5'-cyclic phosphate (cAMP)	14.4	14.8
8-Bromadenosine 3':5'-cyclic phosphate	8.7	5.0
8-Methylthioadenosine 3':5'-cyclic phosphate	12.8	5.1
N <sup>6</sup> -Monobutyryladenine 3':5'-cyclic phosphate	10.9	5.1
2'-O-Monobutyryladenine 3':5'-cyclic phosphate	11.8	5.8
N <sup>6</sup> -Benzoyladenine 3':5'-cyclic phosphate	11.0	5.7
N <sup>6</sup> ,O <sup>2</sup> -Dibutyryladenine 3':5'-cyclic phosphate	10.5	5.8

\* The complete reaction mixture (0.1 ml) contained 40 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 0.1 M KCl, 1 mM MgCl<sub>2</sub>, 1 mM GTP, 50 pmoles of <sup>35</sup>S-Met-tRNA<sub>i</sub> (4500 cpm/pmol) and 10  $\mu$ g of eIF-2 from either interferon treated cells or untreated cells. 12  $\mu$ M cAMP and the same concentration of its derivative compounds were added to the separated reaction mixtures and incubated for 10 min at 37°. The eIF-2 activity was determined as described in Materials and Methods.

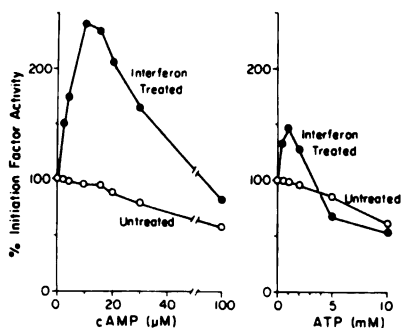


FIG. 1. Effect of cAMP and ATP on the eIF-2 activity from interferon-treated and untreated cells. The eIF-2 from cells treated with interferon (300 units/ml) and untreated cells prepared as described in Materials and Methods. The activity of eIF-2 (10  $\mu$ g protein) was assayed in the presence of either cAMP (left) or ATP (right). The eIF-2 activity was plotted as % of initial activity. 100% of eIF-2 activity corresponds to the activity to 10  $\mu$ g of eIF-2 from interferon-treated and untreated cells, respectively. The eIF-2 activity from interferon-treated (●) and untreated cells (○).

compared with its absence (Table II). The enhancing effect of Mg<sup>2+</sup> also occurred with Mn<sup>2+</sup>, but not with Ca<sup>2+</sup> (data not shown) reversal of interferon-impaired eIF-2 activity by cAMP, ATP and either Mg<sup>2+</sup> or Mn<sup>2+</sup> strongly suggests an enzymatic control of the eIF-2 activity related to phosphorylation by protein kinases.

The specific involvement of interferon was established as follows. Using an eIF-2 preparation from untreated cells, no effect of cAMP and ATP on the eIF-2 activity was observed (Fig. 1). Similarly, eIF-2 preparations from cells treated with heterologous human leukocyte interferon (300 units/ml) at levels which did not induce antiviral activity in mouse L cells (18), or treated with both mouse interferon (300 units/ml) and either actinomycin D (98% inhibition of cellular RNA synthesis) or cycloheximide (93% inhibition of cellular protein synthesis) which inhibit the action of interferon on the cells (19, 20) were not affected by cAMP and ATP (data not shown).

TABLE II. EFFECT OF ATP, cAMP, dsRNA AND AMINOPURINE ON eIF-2 ACTIVITY IMPAIRED BY INTERFERON.<sup>a</sup>

Addition	<sup>35</sup> S-Met-tRNA <sub>f</sub> in ternary complex	
	+Mg <sup>2+</sup>	-Mg <sup>2+</sup>
	pmol	pmol
	4.9	5.2
ATP	7.9	5.9
cAMP	12.8	8.1
cAMP + 1 mM ATP	14.8	8.7
dsRNA	4.7	4.8
11 dsRNA + 12 μM	5.1	5.0
P + 1 mM ATP		
11 dsRNA	4.9	4.9
11 dsRNA + 1 mM ATP	2.0	4.5
11 dsRNA + 12 μM	2.4	4.6
P + 1 mM ATP		
11 dsRNA + 2 mM	4.7	5.0
aminopurine + 1 mM ATP		
aminopurine	5.1	5.0
Aminopurine + 12 μM	5.5	5.3
P + 1 mM ATP		

<sup>a</sup> complete reaction mixture (0.1 ml) contained 40 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 0.1 M KCl, GTP, 50 pmoles of <sup>35</sup>S-Met-tRNA<sub>f</sub> (4500 cpm/μmol) and 10 μg of eIF-2 from interferon treated or untreated cells. The reaction mixture was incubated for 30 min at 37°. The effect of ATP, cAMP, dsRNA and aminopurine on the eIF-2 activity was examined in the presence of 1 mM MgCl<sub>2</sub>. Poly (rI):poly (rC) was 1:1 (22) and used as a dsRNA.

effect of dsRNA and aminopurine on the activity from interferon treated cells. The effect of cAMP and ATP requirements for the increase in interferon-impaired eIF-2 activity suggest the possibility that the reversal reaction may be associated with protein kinase(s). To test the possibility the effect of protein kinase inhibitors (aminopurine and high concentrations of dsRNA (7, 21)) on the reversion of interferon-impaired eIF-2 activity by both cAMP and ATP was examined. When the eIF-2 from interferon treated cells was incubated with both cAMP (12 μM) and ATP (1 mM) in the presence of Mg<sup>2+</sup> (1 mM), the increased level of eIF-2 activity was close to that obtained from untreated cells without any chemicals (Tables I and II). This increase in activity was not observed under conditions of inhibition of protein kinase activity by dsRNA (5 μg/ml) or aminopurine (2 mM). The concentration of dsRNA and aminopurine used inhibited more than 95% of protein kinase activity (histone phosphorylation with γ-<sup>32</sup>P-ATP) which was also

present in the eIF-2 preparation from interferon-treated cells but did not significantly affect the eIF-2 activity (Table II). However, the activity of eIF-2 from interferon-treated cells was further reduced when incubated with a low concentration of dsRNA (50 ng/ml which activates certain protein kinases) in the presence of both ATP and Mg<sup>2+</sup> (about 78% inhibition). The effect of a low level of dsRNA was not observed in the eIF-2 preparation from untreated cells and was prevented by the addition of aminopurine (2 mM).

These results suggest that the increase and decrease of activity of eIF-2 from interferon-treated cells by cAMP and low levels of dsRNA in the presence of ATP, respectively, may involve protein kinases which are sensitive to both aminopurine and high concentrations of dsRNA, and requires Mg<sup>2+</sup> for optimum enzyme activity.

**Discussion.** We have presented indirect evidence which suggests that the interferon-induced mechanisms for regulation of eIF-2 activity have general similarities to those of polypeptide chain initiation induced by HRI in reticulocyte lysates (2-9). These similarities are that: (a) Both inhibitions are specifically overcome in the presence of suitable concentrations of cAMP; (b) the effect of cAMP is stimulated by the addition of either Mg<sup>2+</sup> or Mn<sup>2+</sup>; (c) restoration of activity by both cAMP and ATP are prevented by protein kinase inhibitors such as aminopurine (2-5 mM) or high concentrations of dsRNA (5-10 μg/ml); and (d) low concentrations of dsRNA (10-200 ng/ml) stimulates both inhibitions. Thus the control of eIF-2 activity in both systems may be due to specific phosphorylations of eIF-2 by different protein kinases.

Dissimilarities of the two systems are that preincubation of impaired eIF-2 from interferon-treated cells with cAMP and GTP, which has an effect in reticulocyte lysates, has no effect in our system (data not shown) and ATP (1 mM) which is ineffective in the reticulocyte lysates partly reverses the eIF-2 activity of interferon-treated cells. Moreover, low levels of cAMP which reverse the eIF-2 activity of interferon-treated cells have no effect on the HRI-induced reduction of eIF-2 activity (7). Thus, the two systems have major similarities but may not be entirely comparable.

If protein kinases actually are involved in these processes it is possible that the cAMP and ATP requirements for the reversion of interferon-impaired eIF-2 activity occurs in conjunction with either preexisting protein kinase or with interferon-induced new or increased protein kinase synthesis. Elevation of eIF-2 activity by both cAMP and ATP in the presence of  $Mg^{2+}$  does not occur in the eIF-2 preparation from untreated cells, from cells treated with both actinomycin D and interferon, or from cells treated with heterologous human leukocyte interferon (data not shown). Moreover, low concentrations of dsRNA (10–200 ng/ml) also do not enhance the inhibition of eIF-2 activity from untreated cells (data not shown). Therefore, it seems more likely that if protein kinases are involved they are either newly induced or increased by interferon. This aspect is under active study.

Previous reports indicate that the addition of low concentrations of dsRNA (10–200 ng/ml which stimulates certain protein kinases) to cell extracts from interferon-treated cells induces: (a) enhanced inhibition of viral protein synthesis (10); (b) dsRNA-dependent protein kinase mediated synthesis of a low molecular weight inhibitor (LMW-inhibitor) which directly inhibits viral mRNA translation in cell-free system (11–13); (c) phosphorylation of ribosomal and cellular proteins (14–16); (d) activation of uncharacterized protein kinases (14–16); and (e) activation of an endonuclease which digests viral mRNAs faster than those of host mRNAs in interferon treated cells (17). This activation of protein kinase and the protein phosphorylation may explain our finding that the activity of eIF-2 from interferon-treated cells is strongly decreased by the addition of low concentrations of dsRNA (50 ng/ml) in the presence of both ATP (1 mM) and  $Mg^{2+}$  (1 mM). Although it is not clear why the activity of eIF-2 from interferon treated cells is differentially affected by different concentrations of dsRNA (50 ng/ml and 5  $\mu$ g/ml), there are several possibilities which include activation or inhibition of the same or different protein kinases by the different concentrations of dsRNA (7).

**Summary.** Interferon treatment of mouse L cells causes the reduction of activity of initiation factor (eIF-2) which forms a ternary complex with Met-tRNA<sub>i</sub> and GTP. The activity of eIF from the cells treated with interferon was specifically increased when incubated with 12  $\mu$ M cAMP, but no effect of cAMP on the eIF-2 activity from untreated cells was observed. ATP (1 mM) also slightly increased the interferon-impaired eIF-2 activity. The restoration of activity of eIF-2 from interferon-treated cells was completely prevented by the addition of inhibitors of protein kinases (either aminopurine (2 mM) or a relatively high concentration (5  $\mu$ g/ml) of dsRNA (poly rI:poly rC)) without a direct effect on normal eIF-2 activity. However, low concentrations of dsRNA (50 ng/ml) which activate certain protein kinases, strongly stimulated the reduction of eIF activity induced by interferon. Taken together, these observations suggest that different protein kinases may be involved in the interferon-induced reduction of eIF-2 activity and the restoration of interferon-impaired eIF-2 activity.

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## The Isoproterenol Stress Test in Unanesthetized Atherosclerotic Rabbits (40370)

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Cardiac stress testing is a necessary diagnostic prelude to selective coronary angiography. The most prevalent stress test utilized is exercise performed on a treadmill or bicycle ergometer. There are situations, however, in which exercise stress testing cannot be performed due to physical or psychological limitations (1). It is also difficult, at times, to obtain stable electrocardiographic (ECG) traces due to movement artifacts. Atrial pacing at rapid heart rates has also been utilized as a cardiac stress test (2, 3) but has the disadvantage of requiring cardiac catheterization.

Isoproterenol infusion has been reported to cause S-T segment and T wave changes indicative of ischemia in patients with coronary artery disease but not in normal individuals (4). In addition it has recently been reported to be more reliable than the Masters two-step (5) and as reliable as treadmill exercise (1) in predicting the presence of coronary artery disease in man. Because of its simplicity and great sensitivity it has been suggested that the "isoproterenol stress test" would appear to have a useful role in the clinical assessment of coronary artery disease (1). We have investigated the response to this isoproterenol stress test in an atherosclerotic rabbit model previously described (6, 7). This model is pathophysiologically similar to the patient with coronary artery disease, demonstrates a similar ECG response to the stress of atrial pacing, and responds similarly to pharmacological interventions.

**Methods.** Twenty-three male New Zealand white rabbits, weighing approximately 2 kg, were fed a standard laboratory chow pelleted with 2% cholesterol for 14-16 weeks. At that

time surgical preparation of the animals was carried out as previously described (6). Briefly, a 14 G polyvinyl chloride catheter was implanted in the right external jugular vein under local anesthetic (procaine HCl) for subsequent infusion of isoproterenol. At least 24 hr later, the unanesthetized rabbits were lightly restrained on their backs. Surface leads were placed over the spine and sternum for recording the ECG on a Brush recorder (Mark 260) at a standard sensitivity of 1 mv/cm and on magnetic tape for computer analysis of S-T segment response. A solution of isoproterenol was infused continuously at a rate of 0.2 cc/min for 10 min using a commercial infusion pump (Sage Instruments, Model 255-1). The initial concentration of isoproterenol was such that a dose of 1  $\mu\text{g/kg/min}$  was delivered. If S-T segment depression (i.e. at least 1 mm difference from control) was not seen after 3 min of infusion at this concentration, the infusion was stopped and the concentration of isoproterenol was increased to deliver 2-3  $\mu\text{g/kg/min}$ . The rate of infusion and volume of fluid infused was always constant, however. Propranolol (Inderal) (0.01-1.0 mg/kg,  $n = 6$ ), nitroglycerin (100  $\mu\text{g/kg}$ ,  $n = 9$ ), dipyridamole (Persantin) (250  $\mu\text{g/kg}$ ,  $n = 8$ ) or saline (0.2 cc/min,  $n = 5$ ) was injected intravenously into the marginal vein of the left ear during the fifth or sixth min of the isoproterenol infusion to determine the effects on isoproterenol-induced heart rate and S-T segment depression. Dosages were selected based upon previous experience with these compounds in the paced rabbit model (6, 7).

Statistical analysis of the effects of propranolol, nitroglycerin (GTN) and dipyridamole were determined using Students *t* test (8).

**Results. Effects of isoproterenol infusion.** Heart rate began to increase almost immediately after the start of isoproterenol infusion and reached a steady-state value within 2 min as did ischemic S-T segment changes. Two

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t S-T segment changes occurred during isoproterenol infusion: S-T segment depression and S-T segment elevation. This paper deals only with the S-T segment depression because this is the primary electrocardiographic response to stress in anginal patients. The occurrence of S-T segment elevation will be investigated in future experiments. The attempt to explain the hemodynamics and pharmacology involved.

Examples of S-T segment depression and T-wave inversion in response to isoproterenol infusion can be seen in Figs. 1 and 3. In each case, isoproterenol infusion caused a significant tachycardia which was accompanied by a significant S-T segment depression. An indication of the reproducibility of the ECG response in response to a given isoproterenol dose in a given rabbit is evident in Fig. 1. The tracings are from the same rabbit on two different experimental days. In both instances, there is a classic, sagging S-T segment depression and T-wave inversion in response to isoproterenol infusion.

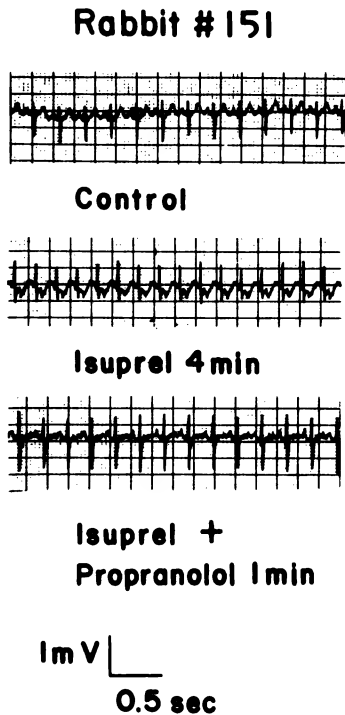


FIG. 1. ECG tracings demonstrating the reversal by propranolol (0.1 mg/kg) of the tachycardia, ischemic S-T segment depression and T-wave inversion caused by isoproterenol infusion.

**Effects of propranolol on isoproterenol-induced ECG changes.** The effects of a dosage of 0.1 mg/kg of propranolol on isoproterenol-induced tachycardia and ECG changes in one rabbit are shown in Fig. 1. Isoproterenol infusion caused an increase in heart rate from a control value of 240 to 310 beats/min, S-T segment depression and T-wave inversion. One min after intravenous injection of propranolol the heart rate decreased to 260 beats/min, and the S-T segment depression and T-wave inversion disappeared although the isoproterenol infusion continued. This is a typical response to a  $\beta$ -blocking dose of propranolol.

A dose-response relationship for propranolol can be seen in Fig. 2, in which the effects of three dosages of propranolol on heart rate and S-T segment depression are compared with those of saline (five animals per group). Propranolol did not significantly reduce heart-rate or S-T segment depression at the 0.01 mg/kg dose but did at the two higher doses.

**Effects of nitroglycerin and dipyridamole on isoproterenol-induced ECG changes.** Nitroglycerin was effective in reversing isoproterenol-induced S-T segment depression whereas dipyridamole had no beneficial effect. In fact, dipyridamole frequently caused

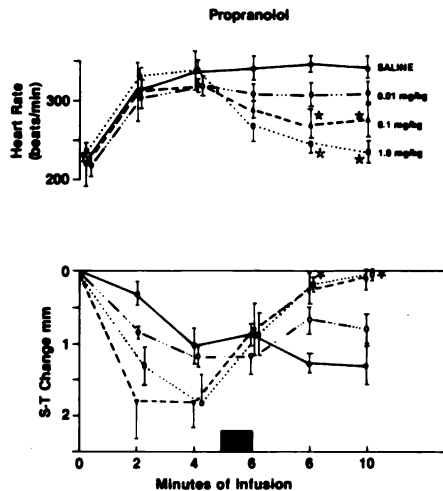


FIG. 2. Dose-response relationship of the effects of propranolol on isoproterenol-induced tachycardia and S-T segment depression (mean  $\pm$  SEM). Asterisks in this and subsequent figures indicate significant differences ( $P < 0.05$ ) when compared with the value at the fourth minute of isoproterenol infusion.



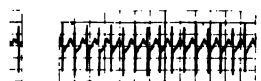
# ISOPROTERENOL STRESS TEST

the S-T segment depresses a typical response to isoproterenol in the same animal on several days. The response to isoproterenol was the same on both days, with a peak heart rate of 390 beats/min and a sagging S-T segment developing inversion.

Administration of isoproterenol during isoproterenol reversed the ischemic S-T segment depression without affecting the tachycardia. Dipyridamole, on the other hand, further depression of the S-T segment (to 1.6 mm) without changing the heart rate.

The effects of nitroglycerin on isoproterenol-induced S-T segment changes is shown in Figure 4. The heart rate and S-T segment changes during isoproterenol infusion were the same for all animals. Neither drug affected the isoproterenol-induced tachycardia. Following administration of the mean S-T segment depression was reduced from 1.3 to 0.4 mm, while dipyridamole tended to exacerbate the depression (to 1.6 mm).

*Effects of Isoproterenol Infusion*



Isuprel 8 min  
Persantin + 3 min

V  
0.5 sec

Figure 5 showing reversal of isoproterenol-induced S-T segment depression by nitroglycerin (Persantin) and the isoproterenol-induced tachycardia from the same animal on several days.

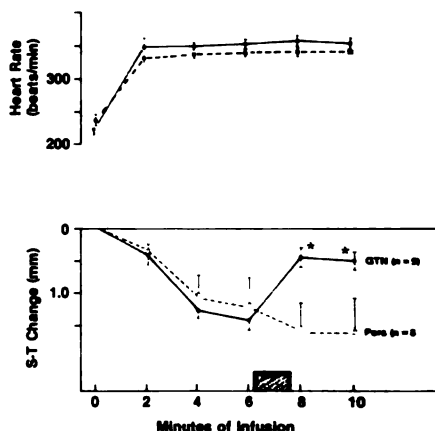


FIG. 4. Summary of the effects of nitroglycerin and dipyridamole on isoproterenol-induced heart rate and S-T segment changes (mean  $\pm$  SEM). The only significant effect was the decrease in S-T segment depression following nitroglycerin (100  $\mu$ g/kg) administration.

*Isoproterenol Infusion.* Infusion of isoproterenol intravenously to unanesthetized atherosclerotic rabbits caused ischemic S-T segment depression and T-wave inversion similar to that seen when patients with coronary artery disease are similarly stressed (1, 4, 5, 9). Electrocardiographic S-T segment depression is thought to be a manifestation of subendocardial ischemia (10). The major portion of total coronary blood flow, and all of subendocardial blood flow, occurs during diastole (11). Therefore the supply of blood, and thus oxygen, to the subendocardium depends upon the diastolic perfusion pressure, the coronary resistance, and the duration of diastole. The diastolic blood pressure (afterload) is also a major determinant of oxygen consumption, and its net effect on myocardial oxygen balance is determined by the relative contribution to coronary flow (oxygen supply) and cardiac work (oxygen demand). Infusion of isoproterenol causes tachycardia and a positive inotropic effect thereby greatly increasing myocardial oxygen consumption. The presence of the tachycardia reduces the duration of diastole and thus the time during which subendocardial coronary flow can occur. Diversion of blood flow away from the subendocardium by isoproterenol has been reported (9). Thus, during infusion of isoproterenol to these animals with compromised coronary circulation, the highly susceptible subendocardium performs more work at a

oxygen cost in the presence of decreased blood supply and becomes ischemic. Ischemia is manifested by electrocardiographic S-T segment depression.

*Effects of propranolol on isoproterenol-induced ECG changes.* Presumably the ischemic segment depression elicited by isoproterenol infusion in these experiments was secondary to heart-rate and contractility increases brought about by beta-receptor stimulation. As might be expected from a complete pharmacological antagonism, increasing doses of propranolol caused a dose-dependent inhibition of the effects of isoproterenol infusion (Fig. 2). The results of the present study have a clinical corollary in the work of Ushiyama *et al.* (5) who reported that propranolol (5 mg, iv) completely suppressed the isoproterenol-induced heart rate increase and ischemic S-T segment depression in each of the anginal patients tested.

Propranolol is a clinically effective antianginal agent (12), the major beneficial effects of which are attributed to the reduction of heart rate and cardiac work due to blockade of  $\beta$ -adrenergic stimulation by endogenous catecholamines. Its action in this animal model with coronary artery disease (13) mimics clinical effects.

*Effects of nitroglycerin and dipyridamole on isoproterenol-induced ECG changes.* While there are differences of opinion regarding its mechanism of action, the efficacy of nitroglycerin in the therapy of angina pectoris is beyond question. Dipyridamole, however, has been shown to have only a benign coronary vasodilatory effect that has no significant therapeutic value when compared to placebo under double-blind conditions and may exacerbate the patient's angina (14). The vasodilation caused by dipyridamole is believed to be due to its inhibition of adenosine deaminase and cellular uptake of adenosine, and it therefore might be expected to be effective only in vascular beds that are not fully dilated because of ischemic constriction. This is borne out by its tendency to cause "coronary steal" from ischemic regions. Nitroglycerin also causes vasodilation in all types of vascular beds, however, its peripheral vasodilatory effects, particularly in the coronary system, are felt to be chiefly (16, 17) and not solely (18) responsible for its antianginal

effects. Its dramatic effect on isoproterenol-induced S-T segment depression in the present experiments suggests that this mechanism of venous pooling was operative since it is difficult to imagine coronary vasodilation being less than maximal under the conditions imposed by isoproterenol infusion in these animals with compromised coronary circulation. It is highly unlikely that the  $\beta$ -adrenergic blocking effects of nitroglycerin (19) were in any way involved since there was no effect on isoproterenol-induced tachycardia. In addition, the doses of nitroglycerin used to demonstrate  $\beta$ -adrenergic blockade were 30 times those used in this study.

Comparison of the effects of nitroglycerin and dipyridamole on ischemic S-T segment depression in this study shows a good correlation with clinical results, i.e. nitroglycerin has a beneficial effect whereas dipyridamole does not. The isoproterenol stress test appears to be a useful adjunct to atrial pacing in this experimental model of angina pectoris as well as in the clinical setting.

*Summary.* Isoproterenol infusion was employed as a cardiac stress test in unanesthetized, atherosclerotic rabbits. In addition to tachycardia, isoproterenol infusion caused ischemic S-T segment depression of the electrocardiogram. Propranolol (0.1 and 1 mg/kg, iv), given during isoproterenol infusion, reversed the tachycardia and S-T segment depression. Nitroglycerin (100  $\mu$ g/kg, iv) reversed the ischemic S-T segment depression but did not affect the tachycardia. Dipyridamole (250  $\mu$ g/kg) tended to exacerbate S-T segment depression, and had no effect on the tachycardia. The effects of nitroglycerin and dipyridamole in these animals correlated well with clinical results, i.e. nitroglycerin had a beneficial effect whereas dipyridamole did not. We conclude that the stress of isoproterenol infusion is as useful as atrial pacing in this experimental model of angina pectoris.

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## Characterization and Evidence for a Precursor in the Formation of Plasma Antinatriuretic Factor (40371)

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Considerable evidence supports the existence of a humoral natriuretic factor which regulates the renal response to extracellular volume (ECFV) expansion (1). Reports from this laboratory have demonstrated a plasma of ECFV expanded (VE) which is natriuretic in rats and inhibits sodium transport (antinatriuretic activity) in the distal urinary bladder (2, 3), a biological function of the distal renal tubule. A similar activity has been reported by other laboratories in plasma of VE humans (4), in urine of VE rats (5), and in renal tissue of VE rats (6). We now wish to report the partial purification of this substance by high pressure liquid chromatography and the development of a bioassay for it. The data suggest the existence of a low molecular weight, acidic peptide which provides evidence of its formation from a precursor molecule.

**Materials and methods.** Blood samples (150  $\mu$ l) were obtained from the jugular vein of conscious (H) and VE dogs as previously described (3). Blood was collected in heparinized syringes and handled according to two different protocols.

**Group I.** Blood was centrifuged at 2500 rpm for 20 min, the plasma was aspirated and stored at  $-4^{\circ}$  until processing by high pressure liquid chromatography. The time interval between collection and processing varied from several days to 2 months. Eighteen to twenty milliliters of plasma were eluted on a Biogel P-2 (medium) column,  $2 \times 95$  cm, with 0.1 M acetic acid as eluant. Ten milliliter fractions were collected automatically and assayed for uv absorbance at 280 nm, and electrical resistance to detect the salt peak. Fractions eluting immediately after the peak (Fraction IV) was lyophilized and stored at  $-70^{\circ}$  for high pressure liquid chromatography. Biogel Fraction IV was redissolved in 300  $\mu$ l of 0.05 M HCl and separated on high pressure Partisil SCX (cation-exchange column (Whatman Inc., Clifton, NJ)

under a protocol previously described (7). Four minute fractions ( $\sim 1$  ml) were collected in a fraction collector.

Peptides in the column effluent were detected by a discontinuous stream-splitting valve coupled to a fluorescamine detection system, as previously reported (8). The valve loops were calibrated to provide one percent of the column effluent for detection, while 99% was diverted to a fraction collector. Column effluent fractions comprising each peak seen on the recorder were pooled and freeze dried. The residue was redissolved in amphibian Ringer and assayed for antinatriuretic activity (AA) as previously described (3). AA is reported as percent decrease in short circuit current (SCC).

**Group II.** In Group II, blood from each dog was split into equal, paired samples and processed by two different methods. In Group II (a) (rapid processing) blood was collected in iced, heparinized syringes containing 50 nmoles bacitracin (an enzyme inhibitor), and centrifuged at 10,000 rpm at  $4^{\circ}$  for 5 min. The plasma was quickly aspirated, acidified to pH 5.0 with 10% acetic acid, and placed in a boiling water bath for 20 min. The total elapsed time from drawing of blood to placement in the water bath was 15 min. In Group II (b) (slow processing), blood was drawn without bacitracin and centrifuged at 2500 rpm for twenty minutes at  $4^{\circ}$ . The plasma was aspirated and allowed to sit at room temperature for approximately 30 min, then acidified and boiled as in Group II (a). In Group II (b), the elapsed time from drawing of blood to boiling was approximately 60 minutes. Then the extract was centrifuged and the supernatant removed and stored at  $-70^{\circ}$ . Twenty-five milliliter supernatant samples were eluted on Biogel P-2 as described above for Group I. Biogel P-2 Fraction IV was then lyophilized and processed on Partisil SCX as described above for Group I.

In most instances, bio-assays were per-

formed on randomly selected bladders. However, in Group II samples, two of the six pairs of assays were performed on paired hemibladders from the same toad and one pair of assays on the same bladder section.

Ten percent of each SCX fraction was used for the reverse-phase peptide analysis of Gruber *et al.* (9). The sample (200  $\mu$ l) was diluted with 300  $\mu$ l of 0.05 M sodium phosphate buffer (pH 7), reacted with fluorescamine, and the peptide-fluorophors separated on a Partisil ODS-2 column (Whatman Inc., Clifton, NJ). The peptide-fluorophors were eluted with a 5–30% acetone:water gradient.

**Results.** AA in plasma extracts of VE dogs was consistently found in a post salt u-v absorbing peak (peak IV) on Biogel P-2 chromatography in Group I samples as previously reported (2). Partisil SCX chromatography of Biogel Fraction IV resulted in the appearance of several fluorescamine-reactive peaks V (Fig. 1). No consistent difference could be observed in the chromatograms of VE and H plasma. These peaks did not contain intrinsic fluorescence (at 390 nm excitation - 475 nm emission). This was shown by turning off the fluorescamine pump in the preparative monitoring system and observing the absence of any peaks on the recorder. AA was only found in the void volume peak (Fraction I) of the VE extracts (Fig. 1 and Table I). There was negligible AA in the fraction (II) immediately after the void volume (Table I), nor did any other column fraction contain AA. Since Guggenheim *et al.* (10) reported that ammonia has AA, all samples were analyzed for ammonia. Our "ammonia titration" curve on the toad bladder shows a plateau of AA at -14% between 0.5 mM and 1.5 mM. In Group I and II samples, ammonia concentrations were uniformly less than 0.4 mM, causing trivial degree of AA in our assay.

Aliquots of the Partisil SCX void volume fractions were reacted with fluorescamine at pH 7, and the resulting peptide-fluorophors separated on a Partisil ODS (reverse-phase) column. The pH used for the reaction has been shown to maximize the fluorescence of peptides, while minimizing the fluorescence of amino acids (11). A group of peaks, consistently found after the void volume peak in VE samples, was used as a marker permitting a blind assay for AA. The fluorescence of

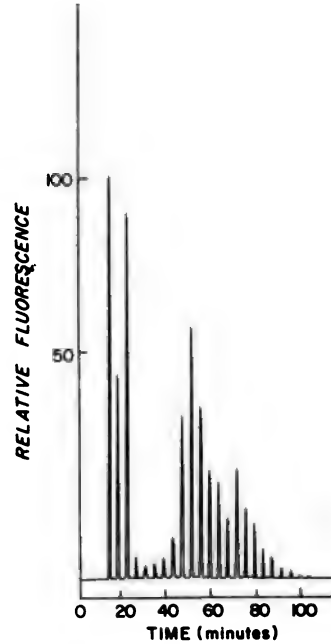


FIG. 1. Partisil SCX chromatogram of Biogel Fraction IV from an ECFV expanded dog. AA is found in the first two sampling periods (void volume). E line in the discontinuous tracing represents the fluorescence in one test tube in the fraction collector.

Group I		
Sample #	Fraction 1 AA %	Fraction 2 AA %
Expanded		
1	-27	-16
2	-21	-11
3	-23	+9
4	-34	+19
5	-23	—
6	-26	-2
mean	-26	0
SE	1.89	6.40
Hydropenic		
1	-10	
2	+31	
3	-9	
4	-27	
5	-5	
mean	-4	
SE	9.53	
P <sup>b</sup>	<.02	

<sup>a</sup> Fraction 1 = void volume of HPLC column. Fraction 2 = fraction immediately after void volume.

<sup>b</sup> Significance of difference between volume expanded and hydropenic groups.

these peaks was due entirely to their reaction with fluorescamine. The reverse-phase chemical assay correlated with the toad bladder assay in 80% of the samples tested.

To determine whether enzymatic degradation reduced the yield of our factor, we collected a series of plasma samples to which acitracin had been added. To our surprise, this resulted in a decrease in activity in the 'E' samples, and reverse-phase chromatograms which resembled H samples.

Accordingly, a study was performed in which plasma samples from VE dogs were divided into paired aliquots (Group II a and b). Bacitracin was added to one-half of the sample, which was processed rapidly with immersion in boiling water to stop further enzymatic activity and to precipitate proteins. The other half was processed more slowly without bacitracin, and boiled after 60 minutes. The conditions under which the slowly processed samples were handled approximated those by which the samples in Group I were handled, with the exception that Group I was not boiled.

The results, seen in Table II, clearly show that the rapidly processed samples have significantly less AA than their slowly processed mate. It is interesting to note that sample 6, which gave the lowest AA, was obtained from the dog with the lowest sodium excretion.

Figure 2 shows a typical reverse-phase

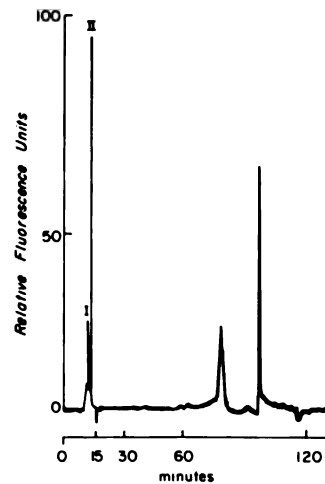


FIG. 2. Reverse-phase chromatogram of a Group II sample. Note peaks I and II.

chromatogram of a Group II plasma extract. The heights of Peaks I and II have a significant correlation with the SCC of Group II (a and b) samples (Fig. 3). H samples did not contain these peaks, as would be expected since they did not possess biological activity.

**Discussion.** An earlier report from this laboratory demonstrated AA in the post salt fraction of plasma from ECFV expanded dogs eluted on Biogel P-2 in 1 M acetic acid (12). The present study shows further purification of the antinatriferic factor in this fraction by high pressure liquid chromatography. The mobility on Biogel P-2 suggests the factor is a low molecular weight molecule. It is excluded from a cation-exchange resin, appears to react with a reagent (fluorescamine) specific for amino groups at a pH which allows only peptides to develop maximal fluorescence, and is formed by enzymatic action. These data suggest that the antinatriferic factor is an acidic peptide of low molecular weight (~500). These results are in accord with the reported characterization of an antinatriferic factor isolated from urine of uremic patients (13).

Reports have indicated the presence of two natriuretic factors in urine of ECFV expanded subjects (1). One factor, which causes natriuresis in rats after a 20-min delay, appears to have a larger molecular weight than a second factor which produces an immediate natriuresis. The low molecular weight factor is antinatriferic, while the higher molecular

TABLE II. GROUP II EXPERIMENTS EFFECT OF PROCESSING TIME ON ANTINATRIFERIC ACTIVITY OF VOLUME EXPANDED SAMPLES.

Sample #	Antinatriferic activity			
	Rapid	Slow	$\Delta$	% <sup>a</sup>
	%	%	%	
1	-10	-22	-12	120
2	-24	-30	-6	25
3	-13	-28	-15	115
4 <sup>b</sup>	-15	-25	-10	67
5 <sup>c</sup>	-16	-30	-14	88
6 <sup>c</sup>	-7	-13	-6	86
mean	-14	-25	-10	83
SE	2.39	2.65	1.59	14
P <sup>d</sup>			<.01	

<sup>a</sup> Difference between rapid and slow samples.

<sup>b</sup> Paired assay performed on same bladder.

<sup>c</sup> Paired assay performed on hemibladders from same ad.

<sup>d</sup> Significance of difference between paired samples by paired *t* test.

## A PRECURSOR FOR ANTINATRIFERIC ACTIVITY

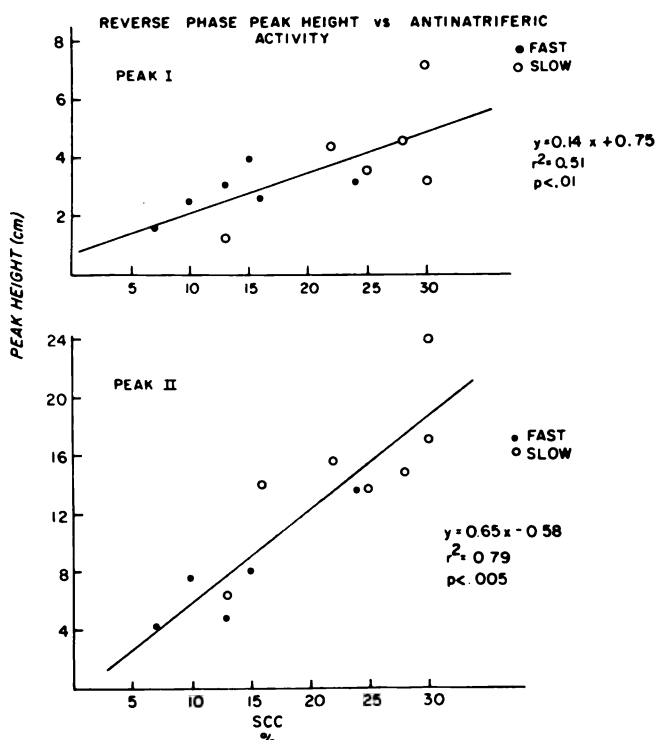


FIG. 3. The plot of height of peaks I and II (from Group II samples) against the SCC of each sample. Note the striking correlation.

weight factor is not, as has been shown in unpublished studies from this laboratory, and others (14). Speculation is that the higher molecular weight factor could be a precursor of the lower molecular weight factor (15).

The correlation of 2 small molecular weight peptides (on reverse-phase chromatography) with SCC in Group II samples (Fig. 3) suggests that they may be the breakdown products of a precursor molecule. This precursor may be the natriuretic, non-antinatriuretic factor previously described (14). These peptides may be responsible for antinatriuretic activity. Because of the high correlation between antinatriuretic activity and peak height, it may be possible to chemically assay for antinatriuretic activity in plasma extracts using the height of Peaks I and/or II.

Our results show that rapid processing of plasma samples reduces the recovery of the antinatriuretic factor. This finding may provide an explanation for previous conflicting reports on the presence of antinatriuretic factor in plasma (1). Our data is the first direct evidence that "natriuretic hormone" is a cas-

cading system. Confirmation of this hypothesis will require isolation and characterization of the precursor substance and its *in vitro* conversion by enzymatic digestion to an effector substance.

**Summary.** Antinatriuretic factor was isolated from VE dog plasma on high pressure liquid chromatography. The use of an enzyme inhibitor while collecting plasma reduced the presence of this factor. A reverse-phase chromatography peptide map revealed 2 peptides whose presence was directly correlated with antinatriuretic activity. The results suggest that antinatriuretic factor is a small acidic peptide, formed from a precursor molecule. Reverse-phase chromatography may prove to be a chemical assay for antinatriuretic factor.

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## Decrease in Renal Perfusion, Glomerular Filtration and Sodium Excretion by Hypoxia in the Dog (40372)

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The present investigation was designed to assess the effect of hypoxia on renal hemodynamics and sodium excretion. Previous studies in dogs have shown conflicting results (1-4). In most studies lowered concentrations of oxygen were administered without controlling ventilation. Presumably, the hyperventilation induced by hypoxia altered blood pH and cardiac output (5); two factors which are known to alter renal function (6-9). Since the pH and  $p\text{CO}_2$  were neither measured nor controlled in these studies altered states of acid-base balance might explain the disparate results. In the present study ventilatory rate was maintained constant so that no changes in  $p\text{CO}_2$  or pH occurred.

Furthermore, in the earlier studies in which hypoxia reduced GFR and RPF (2, 4) it was not determined whether the altered renal perfusion was due to ischemic injury with cell swelling or to a functional and reversible increase in renal vascular resistance. To determine whether hypoxia exerts a direct toxic effect on the renal vasculature the vasodilator acetylcholine was infused into one renal artery during hypoxia and the GFR, RPF, and sodium excretion of this kidney was compared with the non infused kidney.

**Materials and methods.** Studies were performed on 12-18 kg mongrel dogs. Food and water were withheld for 2-6 hr before study. All dogs were anesthetized with pentobarbital, intubated and placed on a Harvard 614 respirator. A polyethylene catheter was inserted into a foreleg vein for infusion of inulin or  $^{125}\text{I}$  iodothallmate (Glo-fil, Abbott Laboratories), and PAH. Plasma inulin was maintained at approximately 20 mg/100 ml and PAH at 2 mg/100 ml. Blood pressure was monitored and blood obtained for clearance determinations from a femoral artery catheter. Both ureters were catheterized near the renal pelvis through bilateral flank incisions. A large polyvinyl catheter for microsphere

injection was advanced through the right axillary or right carotid artery to the aortic root. In the eight animals receiving acetylcholine a hooked 23 gauge needle was placed in the right renal artery and kept open with a saline infusion at a rate of 0.11 ml/min. In four additional animals not receiving acetylcholine a hooked 23 gauge needle was placed in the right renal vein and passed to the hilus for PAH extraction. Arterial blood was obtained initially for PAH,  $p\text{CO}_2$  and  $p\text{O}_2$ . When minor adjustments of the respirator were necessary the dogs were allowed to stabilize an additional 15 min. Acetylcholine was infused at 30-50  $\mu\text{g}/\text{min}$  into the right artery throughout the entire experiment. Following stabilization arterial blood gases were obtained and four 15 minutes control periods were obtained for inulin or  $^{125}\text{I}$  iodothallmate and PAH clearances and sodium excretion. During the second period either strontium 85 or cerium 141 labelled microspheres (15  $\mu\text{M}$ , 3M Company, St. Paul, MI) was given through the aortic catheter. After suspension in 10% dextran,  $10 \times 10^5$  spheres containing about 10  $\mu\text{Ci}$  were rapidly injected and the catheter flushed with 10-15 ml of saline. Following the control period a Heidbrink Kinet-O-Meter (Lundy-Rochester) anesthesia machine with a circle  $\text{CO}_2$  reabsorber was connected in line with the Harvard respirator. To lower the  $p\text{O}_2$  nitrogen was added to oxygen. The addition of nitrogen allowed the arterial  $p\text{O}_2$  to be lowered from 74 to 106 mmHg and maintained between 30 and 40 mmHg. Arterial blood gases were sampled at 5- to 10-min intervals thereafter to insure stability. Following a 30-min hypoxic equilibration period, five to seven 8-min periods were obtained for clearance of inulin or  $^{125}\text{I}$  iodothallmate, PAH and sodium. Microspheres with a different isotope label from those used during control were injected into the aortic catheter. During recovery the anesthesia ma-

was disconnected from the respirator the dog again ventilated with room air. After a 30-min stabilization period arterial blood gases were determined and four recovery periods obtained for inulin, PAH, and PAH.

**Slice preparation for microspheres.** At the beginning of each experiment both kidneys removed, drained of blood and frozen. A coronal slice, 2–5 mm thick, was cut from the center of each frozen kidney. The slice further divided into ten wedges approximately 5 mm wide by cutting perpendicular to the cortical surface. The cortical wedge divided into four equal zones by using a cutting box. While still frozen the slices were rapidly weighed on a Roller-Bal balance and placed in gamma counting. The zones were numbered I (superficial) through IV (juxtamedullary) (10).

**Analytic methods.** Glomerular filtration rate (GFR) was determined by clearance of either inulin (11) or inulin. Inulin concentrations were determined by the diphenylamine method (4). Renal plasma flow (RPF) was estimated from PAH clearance after correcting for extraction. PAH concentrations were determined by the method of Smith (12). Arterial  $pO_2$ ,  $pCO_2$  and pH measured on an IL 113 pH-gas analyzer. Renal plasma flow was measured on an IL 143 flame photometer. Radioactivity was determined in a clear Chicago two channel gamma spectrometer. Fractional blood flow distribution in the renal cortex was calculated from the equation:  $P_z = qz/qt$ ; where  $P_z$  = percent of total flow per g for a given zone uncorrected for volume,  $qz$  = cpm per g of tissues in respective zone, and  $qt$  represents the sum of cpm per g from all four cortical zones (13). A paired  $t$  test was used to determine statistical significance. All values are given as  $\pm$  SE.

**Results. Maintenance of arterial pH,  $pCO_2$  and blood pressure during hypoxia.** There were no significant differences in arterial pH between control ( $7.38 \pm 0.01$ ) or hypoxia at 20 min ( $7.38 \pm .01$ ), hypoxia at 60–90 min ( $7.37 \pm .01$ ) or during recovery ( $7.36 \pm .01$ ). The  $pCO_2$  also remained unchanged during these periods ( $36.5 \pm 1.6$ ,  $36.7 \pm 1.6$ ,  $35.8 \pm 1.8$ ,  $\pm 1.9$ ). Thus, extracellular acid base conditions were maintained constant

throughout the experiment. Mean blood pressure increased from  $111 \pm 2$  mmHg to  $119 \pm 5$  mmHg during hypoxia ( $P < .05$ ) and returned to control level during recovery ( $112 \pm 5$  mmHg).

**The effect of hypoxia on GFR and  $C_{PAH}$ .** The effect of hypoxia on GFR and  $C_{PAH}$  in acetylcholine perfused and nonperfused kidneys is shown in Table I. The GFR of nonperfused kidneys fell  $33 \pm 7\%$  ( $P < .001$ ) during hypoxia. Although the percent change varied, the GFR decreased in each kidney. Restoration of normal oxygen tension caused the GFR to increase in all nonperfused kidneys and the GFR for the entire group had returned to the control level.  $C_{PAH}$  also decreased in all nonperfused kidneys. The mean decrease of  $38 \pm 6\%$  was significant ( $P < .005$ ).

In acetylcholine perfused kidneys GFR did not change during hypoxia. The difference in response of GFR to hypoxia between perfused and nonperfused kidneys is significant ( $P < .005$ ) showing that acetylcholine prevents the hypoxia induced decrease in GFR. The mean 6% decrease in  $C_{PAH}$  of perfused kidneys during hypoxia was not statistically significant. The difference in  $C_{PAH}$  between perfused and nonperfused kidneys is significant ( $P < .005$ ) so it appears that acetylcholine prevents a decrease in  $C_{PAH}$  during hypoxia.

In four additional dogs hypoxia was induced without infusing acetylcholine and PAH extraction determined. During hypoxia GFR fell 42% ( $P < .005$ ) while  $C_{PAH}$  fell 35% ( $P < .025$ ). The renal extraction of PAH was  $0.76 \pm .07$  in controls,  $0.79 \pm .09$  during hypoxia, and  $0.76 \pm .04$  in recovery. These differences were not significant.

**Effect of hypoxia on cortical blood flow.** Fractional blood flow to the renal cortex determined by microsphere distribution is shown in Fig. 1. The fraction of blood flow to inner and outer cortical zones was identical during control and hypoxia. Therefore, the increased vascular resistance caused by hypoxia is evenly distributed throughout the kidney demonstrating that the decrease in whole kidney GFR and RPF are not the result of a redistribution of cortical blood flow. Cortical blood flow distribution in acetylcholine perfused kidneys likewise was un-

TABLE 1. EFFECT OF HYPOXIA ON GFR AND RPF IN DOGS WITH UNILATERAL RENAL ACETYLCHOLINE PERFUSION.

GFR (ml/min)						C <sub>PAH</sub> (ml/min)					
No Ac			Ac			No Ac			Ac		
C	H	R	C	H	R	C	H	R	C	H	R
50.3	40.8	48.8	50.6	46.4	44.7	154.4	135.6	136.3	187.3	174.8	173.1
24.1	21.7	23.1	35.8	46.9	24.4	70.7	57.6	43.4	101.6	104.8	91.6
45.8	28.9	38.9	18.1	16.7	—	95.3	59.2	63.1	64.6	58.9	—
23.6	13.9	20.8	18.2	17.2	15.9	63.6	59.9	91.9	79.1	86.3	109.9
22.1	19.1	25.3	26.8	24.0	25.7	117.6	99.0	96.1	141.5	134.0	119.7
41.1	21.5	32.7	36.0	34.1	31.5	130.4	73.8	92.0	130.6	129.6	101.0
25.1	17.2	21.9	28.5	21.7	21.2	91.2	61.4	66.8	122.3	92.7	78.9
29.9	10.3	48.1	33.8	36.1	39.5	155.6	62.3	175.6	148.9	155.3	172.0
$\bar{x}$	32.8	21.7*	32.5†	31.0	30.4	109.9	76.1*	95.7	122.0	117.1	121.0
SEM	4.26	3.60	4.38	4.03	4.64	13.42	10.48	16.13	15.01	14.53	15.47

GFR, glomerular filtration rate; C, control; H, hypoxia; R, recovery; Ac, acetylcholine perfused kidney; No Ac, kidney without acetylcholine perfusion.  
 \* Comparing C to H;  $P < .01$ ; † comparing H to R;  $P < .025$ .

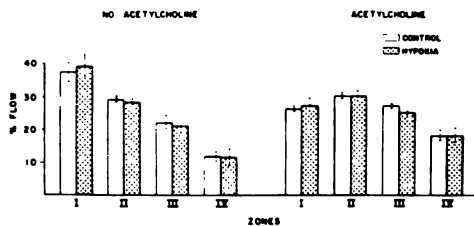


FIG. 1. Fractional blood flow to renal cortex in acetylcholine perfused and nonperfused kidneys. Results represent mean fractional flow  $\pm$  SEM to each cortical zone.

changed by hypoxia. These microsphere data indicate that the maintenance of GFR and RPF with acetylcholine is a result of general renal vasodilatation rather than a redistribution of renal blood flow by the drug.

**The effect of hypoxia on sodium excretion.** Sodium excretion decreased in all the nonperfused kidneys. As shown in Fig. 2 mean sodium excretion in nonperfused kidneys decreased  $65 \pm 9\%$  from control ( $P < .001$ ). By contrast the mean  $8 \pm 10\%$  decrease in sodium excretion of perfused kidneys was not significant. Thus, the decrease in sodium excretion caused by hypoxia is prevented by vasodilatation with acetylcholine.

**Discussion.** Although many studies in dogs have examined the effect of hypoxia on renal function, much of the data are contradictory. Earlier dog studies demonstrated that moderate hypoxia results in either no change (1, 3) or a decrease in GFR (2). Moreover, RPF was reported to be either increased (1), decreased (2, 4) or unchanged (3) and changes in RPF did not always parallel changes in

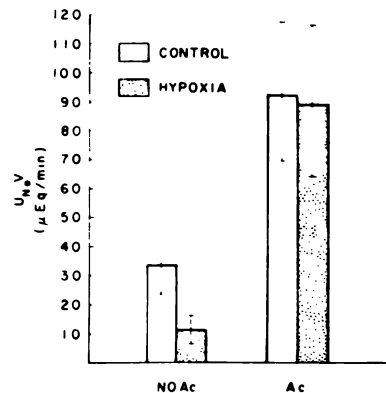


FIG. 2. Effect of hypoxia on sodium excretion. Sodium excretion ( $U_{Na}V$ ) from non acetylcholine perfused kidneys (no Ac) is compared to acetylcholine perfused kidneys (Ac) during hypoxia. The brackets represent  $\pm$ SEM.

GFR. Sodium excretion also varied independently from changes in RPF and GFR and was found to be either decreased (3) or unchanged by hypoxia (2). Alterations in extracellular acid base conditions may at least partially account for the disparate results since pH was neither monitored nor controlled in the studies cited. In most of the previous studies hypoxia was associated with hyperventilation which may have caused a respiratory alkalosis. Alkalosis decreases renal vascular resistance (8) and causes a sodium diuresis (14). There are two studies, however, in which the  $pCO_2$  was maintained constant. In the study of Cosgrove et al (15) most of the animals developed a progressive and unexplained metabolic acidosis. Acidosis

either increase (6, 7, 9) or decrease vas-resistance (8) depending on the degree of hypoxia. Kalyonides (3) maintained  $p\text{CO}_2$  and pH constant while decreasing the  $p\text{O}_2$  from 400 to 44 mmHg in dogs and was unable to detect any change in GFR or RPF. However, raising the arterial oxygen to a level of 160 mmHg significantly decreases RPF. If the initial GFR and RPF in Kalyonides' studies were already reduced by the high level of oxygen, altered renal function induced by acute hypoxia might be undetectable. The present study clearly shows that under acid-base conditions and control oxygenation, renal function is maintained. Hypoxia reduces  $\text{C}_{\text{PAH}}$  and sodium excretion.

Several mechanisms could be responsible for these hypoxia induced effects. First, hypoxia might decrease cardiac output which then leads to a decrease in both RPF and GFR. Although cardiac output was not altered in this study blood pressure remained constant. Also, it has been shown that hypoxia actually augments cardiac output in unanesthetized acutely hypoxic to the same extent as observed in this study (5, 17). It seems unlikely, therefore, that cardiac output was altered.

Second, hypoxia might injure renal tubules resulting in back diffusion of PAH and inulin falsely lowering the calculated GFR and RPF. In various models of acute renal failure PAH (18) and inulin (19) have been shown to diffuse from the renal tubule into the renal venous blood resulting in a decrease in calculated RPF and GFR. However, in the four dogs examined in this study GFR extraction was not altered by hypoxia. The significant back diffusion of PAH accounted for the decrease in calculated RPF. The filtration fraction should have decreased. In addition, maintenance of PAH and inulin clearances during hypoxia in the acetylcholine perfused kidney at a time when these clearances had decreased in the non perfused contralateral kidney also argues against a direct alteration of renal tubular cell integrity. Third, redistribution of cortical blood flow during hypoxia could decrease GFR and cause a functional decrease in RPF and GFR. This has been associated with a redistribution of blood flow away from the outer renal cortex (20). However, redistribution of cortical blood flow, however, cannot account for the hypoxia induced decrease in hemodynamic function found in

this study since fractional flow distribution in the cortex was not altered. Finally, a diffuse increase in renal vascular resistance either functionally or as a result of direct ischemic injury with swelling or spasm of the renal vasculature could explain the decrease in RPF and GFR.

The decrease in RPF when considered with the microsphere data demonstrates that hypoxia diffusely increases renal vascular resistance. Since filtration fraction was unchanged, resistance must have increased in both afferent and efferent arterioles. The return towards control levels of RPF and GFR during recovery demonstrates the reversibility of this increased resistance but cannot truly distinguish between a functional increase in resistance and a direct toxic effect on the renal vasculature resulting from ischemia and cell swelling (21, 22). However, since renal hemodynamic function is maintained during vasodilatation with acetylcholine a direct toxic effect of hypoxia seems unlikely.

The large decrease in sodium excretion seen during hypoxia can be accounted for by the decreases in both renal perfusion and GFR (4). Whether hypoxia exerts a separate effect on renal tubular sodium reabsorption such as altering single nephron filtration fraction (10) was not examined in this study. However, since sodium excretion is maintained with acetylcholine perfusion, it seems unlikely that hypoxia has any direct effect on sodium reabsorption separate from its effect on renal perfusion.

**Summary.** Previous studies of hypoxia induced alterations in renal function have yielded conflicting results. Uncontrolled acid base conditions in most studies may account for this scatter. To eliminate acid-base effects hypoxia was induced in dogs while arterial  $p\text{CO}_2$  and pH were maintained constant for 60–90 min. To test whether the effects of hypoxia were mediated by vasoconstriction or ischemic injury, acetylcholine was infused unilaterally into one renal artery. During hypoxia the GFR and RPF of the kidney not receiving acetylcholine decreased significantly. In the kidney perfused with acetylcholine, however, GFR and RPF did not change. Sodium excretion fell in nonperfused but did not change significantly in acetylcholine perfused kidneys. Using the radiomicrosphere

method, fractional distribution of renal cortical blood flow was found to be unaffected by hypoxia. The data demonstrate that acute hypoxia reversibly decreases GFR and RPF by functionally increasing renal vascular resistance uniformly throughout the kidney and that these changes were associated with a decrease in sodium excretion.

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# Response of the Arterial Wall to Endothelial Removal: An Autoradiographic Study (40373)

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have shown in earlier studies that the al hyperplasia which follows deendothelialization of rabbit aortas is self-limited in the absence of restored endothelial over (1). Thus, the hyperplastic response es a maximum by about two months he insult, although reendothelialization be incomplete for 6 months or longer. Present studies were designed to study ourse of this proliferative response as ted by incorporation of tritiated thymi- into the nuclei of vascular smooth mus- cles, as a function of the interval after lothelialization. The findings indicate he proliferative response subsides with kable rapidity.

*Methods and materials.* Experimental ani- were male New Zealand rabbits, weigh- 4 kg. Surgical procedures were per- d under light sodium pentobarbital an- sia, supplemented with ether as neces- Deendothelialization of the aorta was rmed as previously described (2). oups of three animals were sacrificed at 14, or 28 days after injury; sham oper- animals served as controls. One hour to sacrifice, each animal received 4 mCi [3H]thymidine (New England Nuclear Co.) venously. One-half hour later 4 ml. of s' blue dye were similarly administered by defining blue areas where an endo- l cover was absent (2). Sacrifice and sion fixation was accomplished as pre- ly described (2). Following perfusion, e were excised and quickly cleaned of adventitia by sharp dissection. The ven- rfaces were sliced open, and the arteries d out as *en face* preparations for pho- phy with a Polaroid MP4 camera. This led macroscopic regions which were or white. Tissues were then fixed for l the perfusate before being placed over- in 7% sucrose-0.1 M cacodylate buffer.

Sections of small intestine were obtained to provide controls for [3H]thymidine labeling.

Four segments from each aorta were then chosen for study, selected to provide repre- sentative sampling of both blue and white areas. Particularly in vessels after 2 or more weeks of healing, sections completely stained or free of staining could not be obtained. Accordingly, many sections examined had both blue and white components. Cross sec- tions were excised by sharp dissection and embedded in paraffin. The segments chosen were marked on the Polaroid photographs and given a code number. These were then cut, mounted on slides, and coated with Ko- dak NTB2 emulsion as described by Sprara- gen *et al.* (3). Slides were incubated for 7 days at 4° in the dark, developed, and counter- stained with hematoxylin and eosin.

Labeled cells were defined as those having at least five grains per nucleus. Counts were made microscopically with 450× magnifica- tion and an eye piece reticule micrometer.

Each slide consisted of 4 serial cross sec- tions from the designated areas of each aorta. Counting was done without knowledge of origin of either section, Evans' blue staining, or animal. In each cross section the following variables were evaluated by direct counting: (a) Total number of intimal cells, (b) total number of labeled intimal cells and (c) total number of labeled medial cells. The total number of intimal cells present per cross section ranged from about 30 in control ani- mals to about 500 in 28-day animals. For purposes of more exact localization of labeled cells, the media was divided into four ap- proximately equal levels, with the first being immediately beneath the IEL and the fourth adjacent to the adventitia. The number of labeled cells in each level was then enumer- ated. Finally, the intimal cells oriented on the vessel lumen in a manner similar to endothe-

lial cells were counted, and their location in relation to the blue-white junctions was noted. The total number of medial cells in one section of each slide was then counted. Since the size of the media was constant and did not change following injury, this number of about 500 cells was taken to be an estimate of the total medial cells of each cross sectional area. Data from all sections from all four segments studied in each group of three animals were pooled and tabulated, to give the incidence of labeled cells in each region of the vessel (luminal intima, total intimal, medial levels 1-4) per 1000 nuclei counted  $\pm$  SE of the mean.

Following this initial analysis the code was broken, and the slides were reexamined to evaluate mitotic activity in blue and white areas respectively. This was done by comparing slides with the colors (blue or white) on the photographs which were taken prior to their embedding. Further comparison was made between the cross sections on the slides with the cross sections as they appeared on the uncut remainder in paraffin blocks. Here, the vessel cross section could be plainly seen as blue, white or a mixture. Blue and white areas had their thymidine indices determined as labeled cells per 100 nuclei in the intima or media of each group. Blue and white areas in each cross section were then gauged with respect to intimal thickness by number of cell layers, and actual widths as determined with the optical micrometer. Finally, the presence of [ $^3$ H]thymidine labeling in sections of gut from each animal was ascertained to verify that systemic exposure to the reagent had actually occurred.

**Results.** Identification of labeled cells presented no difficulty, since background counts were negligible. Each animal showed uptake of radioactive label in sections of intestine. The degree of aortic intimal hyperplasia and cell labeling varied among different areas in a single cross section in both blue and white regions. In conformity with previous findings (2), there was also variation in the hyperplastic response along the length of the vessel and among different animals within a group. Whether this reflected an artifact of technique or spontaneous variability is unclear.

Intimal hyperplasia, as measured by the parameters of thickness and total number of

intimal cells per cross section, increased with time after injury (Fig. 1). Mitotic activity, as reflected by [ $^3$ H]thymidine cell labeling, was maximal in the first week post-injury; it rapidly and progressively decreased thereafter (Table I, Fig. 2). Few intimal cells were present 3 days after injury, but those present showed significant mitotic activity. By 6 days a characteristic labeling pattern had become evident: The closer to the lumen the greater the incidence of labeled cells. In the subsequent periods, this relationship was maintained in the face of the reduced proliferative activity, and the luminal cells were the last to return to baseline levels. Of additional interest is the rate of this reduction, which appears to approximate linearity on the semi-logarithmic plot, and gives a halving time of about 3 days.

The relationship between continued proliferative activity and restoration of endothelial cover was evaluated by systematic counting of labeled cells from blue or white areas. Although no significant and consistent differences were observed, it should be noted that the area of reendothelialization up to 2 weeks postinjury was small, thus preventing collection of meaningful data, and no white area counted was more than 2 mm from a blue area. Perhaps this represented lateral diffusion of blood borne mitogens in the vessel wall. Re-endothelialization was more extensive in the 28-day animals, but in these, the labeling index was too low for meaningful comparisons. These data are presented in Table II. Cell proliferation appeared to depend primarily upon time after injury rather than location in blue or white areas. Thus, the thymidine labeling index in blue areas was only slightly higher than in white areas (Table II). Variations in intimal thickness were not associated with differences in mitotic activity in either the intima or media. At 28 days, the vessel walls were almost completely devoid of labeled cells throughout.

**Discussion.** The vascular endothelium, and medial smooth muscle cells, represent relatively stable populations of cells under normal conditions. In the uninjured rabbit aorta the incidence of [ $^3$ H]thymidine labeled cells in the intima and media are about 0.8 and 0.03 per thousand respectively (4). Increased cell turnover and associated mitotic activity

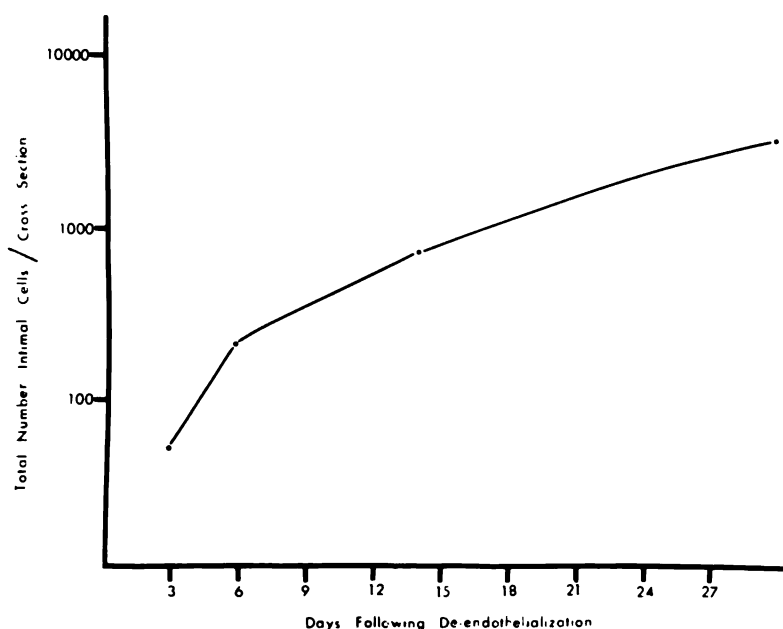


FIG. 1. Intimal hyperplasia following deendothelialization of rabbit aortae from animals sacrificed at varying intervals following endothelial removal with a balloon catheter. Extent of proliferation is expressed as mean number of intimal cells per cross section.

TABLE I. MITOTIC ACTIVITY OF INJURED RABBIT AORTAE.

Location	Incidence of labeled cells per 1000 cells $\pm$ S.E.M.				
	Control	3	6	14	28
Luminal Intima	0.3	160 $\pm$ 35	250 $\pm$ 66	40 $\pm$ 10	2 $\pm$ 2
Total Intima	—	114 $\pm$ 32	138 $\pm$ 32	17 $\pm$ 1	1 $\pm$ .4
Media Level 1	0	12 $\pm$ 7	7 $\pm$ 5	1 $\pm$ 1	0
Media Level 2	0	6 $\pm$ 5	5 $\pm$ 5	0.5 $\pm$ 0.5	0
Media Level 3	0	6 $\pm$ 6	4 $\pm$ 5	0.4 $\pm$ 0.4	0
Media Level 4	0	6 $\pm$ 6	3 $\pm$ 2	0.3 $\pm$ 0.3	0

is seen as a response to various insults in the aortae of rabbits such as atherogenic diets (3), hemodynamic stresses (5), or physical trauma (6).

Medial smooth muscle cells constitute the source of neo-intimal SMC's in the regenerating intima of injured arteries, but cells from the entire breadth of the media are stimulated to divide. Some of these then migrate to the intima and continue dividing. Intimal mitotic activity was typically greater and persisted longer than that of the media. Thus, the majority of neo-intimal cells are generated within the intima itself from a starting pool of SMC's originating in the media. The consequence of these events is preservation of medial thickness in the presence of intimal

hyperplasia.

The present data are in conformity with the earlier observations of Hassler who subjected carotid arteries and aortae to mechanical trauma (7), and with the findings of Webster *et al.* (8) in the rabbit aorta. However, in those earlier experiments, the relationship between moderation of the proliferative response and reendothelialization was not characterized, and no hypothesis was developed concerning the mechanisms involved.

The stimulus for the initial migration and proliferation of the medial smooth muscle cells is presumably based upon the mechanisms suggested by Ross and his colleagues (9), whereby platelets adhering and aggregat-



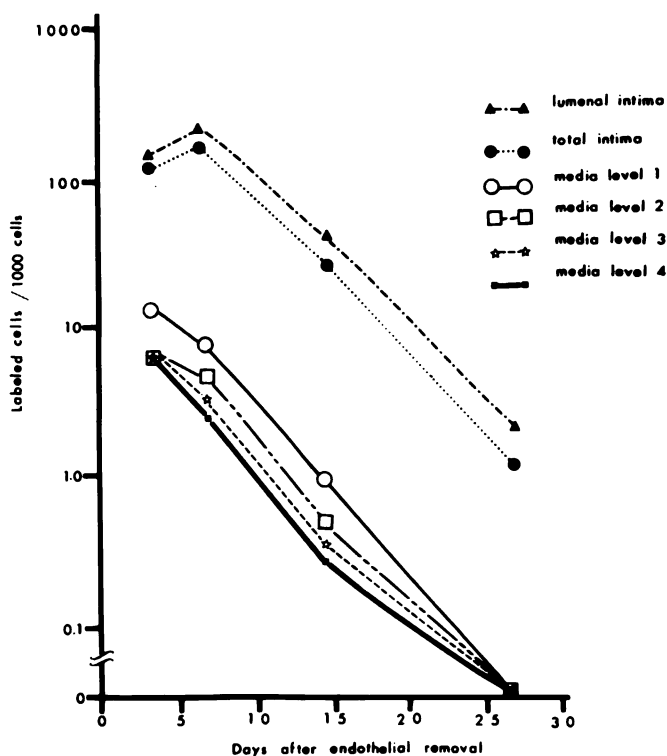


FIG. 2. Intimal medial mitotic activity of rabbit aortae following endothelial removal.

TABLE II. MITOTIC ACTIVITY OF INJURED RABBIT AORTAE TREATED WITH EVANS' BLUE DYE.

Location	Thymidine index (% + S.E.M.) <sup>a</sup>				
	Control	3	6	14	28
Intima:					
Blue areas	—	10.9 ± 5	14.1 ± 6	3.2 ± 2	0.1 ± 0.1
White areas	0	—	13.3 ± 5	2.1 ± 3	0.1 ± 0.1
Media:					
Blue areas	—	1.0 ± 0.5	1.1 ± 0.3	0.1 ± 0.2	0
White areas	0	—	1.0 ± 0.4	0.1 ± 0.2	0

<sup>a</sup> Based on total number of intimal and medial cells present on each cross section.

ing at the sites of exposed subendothelial connective tissue undergo a release reaction, and flood the vessels cells with a mitogenic protein. Several possibilities could account for the transient nature of this response. One of these could be a rapid decline in delivery of platelet mitogen to the vessel, and this would be consistent with the findings of Groves *et al.* (10), who demonstrated a dramatic reduction of platelet turnover shortly after deendothelialization of rabbit aortae by techniques similar to those used by us. Whether platelet turnover is reduced suffi-

ciently to produce the observed changes remains to be determined: We have found well preserved and presumably recently adhering platelet in significant numbers on blue areas for many months after injury (2). Blue areas in the balloon deendothelialized rabbit have been shown to represent the virtual absence of an endothelial cell cover (2). Additionally, even in the presence of sustained turnover, platelets participating at later intervals following injury might undergo a diminished release reaction, and thus make correspondingly less mitogen available. Alternatively,

smooth muscle cells themselves might become progressively less responsive to available mitogen as a consequence of their previous proliferative history. In any event, the phenomenon of the limited hyperplastic response is clearly adaptive: Persistence of the normal proliferative rate would rapidly produce a mass of tissue that would rapidly become occlusive to the lumen whenever macrophage-mediated endothelial loss occurred.

**Summary.** The proliferative response of the non-reendothelialized rabbit aorta was followed by tritiated thymidine labeling. Peak labeling was seen by 6 days after the procedure, with progressively decreasing activity at increasing distance from the lumen. The proliferative response rapidly subsided, so that base-line values were achieved by 14 days after the vessel insult. The decrease in proliferation occurred even in areas which did not re-endothelialize. The mechanism of this moderating response is presently not known, but it appears to have adaptive value in preventing excessive lumen occlusion following vascular injury.

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# Total Salivary Calcium and Amylase Output of Rat Parotid with Electrical Stimulation of Autonomic Innervation (40374)

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A relationship between the kind of autonomic stimulation used to elicit salivary secretion and the concentration of amylase and calcium in the secretion has been demonstrated using an *in vivo* preparation (1-3). Administered autonomic agonists were compared with the effects of direct electrical stimulation of the autonomic nerve fibers (3). The results obtained using the more physiological condition of stimulation (i.e., the nerve) were not identical to those obtained using injected agonists (3). From recent work on the perfused main duct of submaxillary gland where effects of nerve stimulation were compared with effects of administered agonists, major differences between effects of drug administration and nerve stimulation were also observed (4, 5). These findings suggest that the effects observed with injected autonomic drugs may not be equated to effects observed under more physiological conditions of stimulation. When still another modification from the physiological state is introduced (such as use of an *in vitro* system), it is probable that additional discrepancies may become evident. Thus, although the *in vitro* parotid slice model has yielded important information regarding autonomic control of amylase and calcium secretion, it has become evident that the initial postulate of Schramm's group, i.e., that calcium and amylase are packaged together and secreted together across the luminal membrane (6, 7), probably is not true for all conditions of stimulation (3). In fact, recent work has implied that at least two routes for calcium secretion may exist, one involving packaging with amylase and the other may involve secretion of calcium in the saliva without packaging with amylase. To test this hypothesis further, *in vivo* systems

that are more comparable to the *in vitro* ones were employed; these included analysis of gland depletion of calcium and amylase with stimulation and measurement of total salivary output of these two moieties. Disparities between gland depletion and salivary output would be indicative of the importance of other mechanisms. Finally, Schramm (8) also suggested that in the *in vitro* system, any apparent cholinergically induced release of amylase or calcium is actually the result of acetylcholine induced catecholamine release. The validity of this assumption was also examined in the present study, and appropriate adrenergic antagonists were used in conjunction with stimulation of the parasympathetic innervation to test this point.

**Materials and methods.** Female Long-Evans rats used in these experiments were 4-5 months of age, weighed approximately 200 g, and were maintained on lab chow and water *ad libitum*. After 18 hr of starvation, rats were anesthetized with 1% sodium pentobarbital in doses of 50 mg/kg of body wt. The trachea was cannulated to avoid respiratory complications. Collection of saliva was made by application of calibrated micropipettes to the cut orifice of the parotid duct (4). Electrical stimulation of either the auriculotemporal nerve or the superior cervical ganglion was used to elicit flow of saliva from the parotid; square wave pulses of 4 V at a frequency of 20 pulses/sec and 5 msec in duration were delivered to the nerves by a Grass stimulator, SD5. Flow rate was determined by measuring the time required for collection of a given volume of saliva and relating this to gland weight (9). Stimulation and collection of samples were continuous so that not only concentration but total salivary output of calcium and amylase were measured. Calcium concentration was determined on saliva samples by titration of the fluorescent calcium-calcein complex with Ethylene-glycol bis (2 amino

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ether)-NNN'N' tetra acetic acid) (automatic calcium titrator, Fiske Associates, Inc.).

stimulated parotid gland was removed immediately following termination of the stimulation period. The tissue was divided into parts and weighed separately. One part was used for analysis of amylase activity; the other part was put in a crucible, dried overnight and reweighed to determine its wet weight. The dried residue was ashed at 500°C for 12–14 hr. The ash was dissolved in 10 ml of 0.5 N HCl. The resultant solution was neutralized with 0.5 ml of 0.5 N NaOH. Calcium concentration was then determined with an automatic calcium titrator (Fiske Associates, Inc.). Atomic absorption analysis of calcium was also done and the two methods gave essentially the same results.

An unstimulated contralateral control gland was also quickly removed and treated in exactly the same way as the experimental

gland. Amylase activity of appropriately diluted saliva or gland homogenate was determined by methods previously described. Salivary amylase activity was expressed as milligrams of reducing substance formed per milliliter of saliva in a 15 min digestion period at 37°C, whereas glandular amylase was expressed as milligrams of reducing substance formed per milligram of gland in a 15 min digestion period at 37°C. Saliva were obtained continuously during a 60-min period of nerve stimulation. Output of amylase in saliva was also determined.

To rule out the possibility that acetylcholine-mediated release of catecholamines was involved in any of the effects, the  $\alpha$ -adrenergic blocking agent (phenoxybenzamine or phentolamine) and  $\beta$ -adrenergic blocking agent (propranolol or Inderal) were administered in doses of 5 and 3 mg/kg, respectively, 5 min before initiation of nerve stimulation.

**Analysis of data.** All data in the text, tables and figures are expressed as means  $\pm$  SE. Control values were compared with the experimental values by unpaired Student's *t* test. Values were considered to be statistically significantly different if *P* values were less than 0.05.

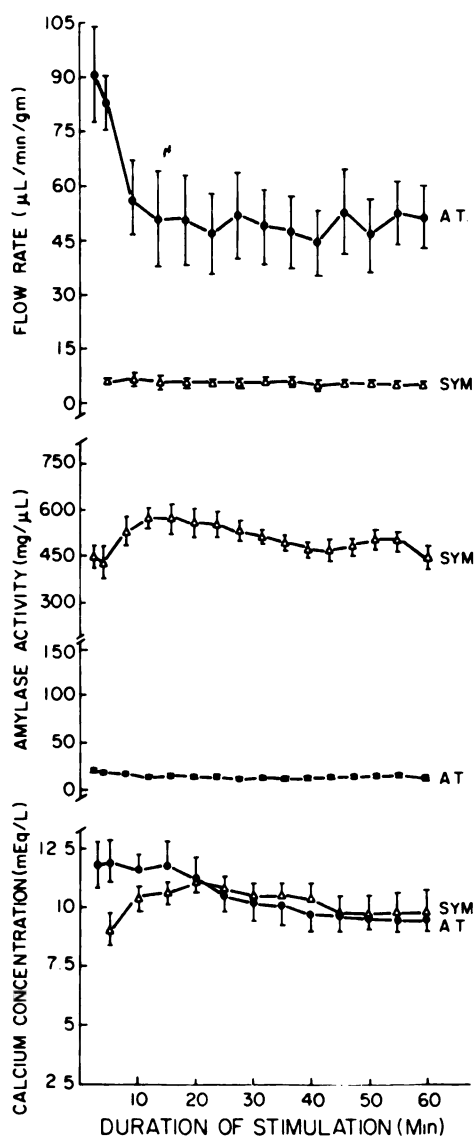


FIG. 1. Time course of flow rate, amylase and calcium concentration of rat parotid saliva during continuous stimulation of the auriculotemporal nerve (AT.) or superior cervical ganglion (SYM.).

**Results.** The data in Fig. 1 summarize the effects of direct nerve stimulation on calcium, amylase and flow rate of saliva from parotid of adult rats. No saliva flow could be observed during the prestimulation period. The calcium concentration of saliva evoked by supramaximal stimulation of the auriculotemporal nerve was initially high (11–12 mEq/l) and remained high (10 mEq/l) during a 60-min period of continuous stimula-

tion. Calcium concentrations of saliva evoked by stimulation of the superior cervical ganglion were initially somewhat lower (9–10 mEq/l), but reached levels similar to those induced by cholinergic stimulation within 20 min and remained at these levels thereafter.

Flow rate under the two conditions of stimulation differed markedly from each other. It was very high initially with cholinergic nerve stimulation (0.105  $\mu$ l/min/mg of gland) but within 10 min fell to levels of about 0.06–0.05; these levels were then maintained for the 60 min of stimulation. On the other hand, flow rate with sympathetic nerve stimulation was initially very low (0.1  $\mu$ l/min/mg) and remained at this level for the 60 min period of stimulation.

Amylase levels were also consistently (initially and thereafter) very low (20 mg/ $\mu$ l of saliva) with cholinergic nerve stimulation. However, with stimulation of the sympathetic nerve, while initial values were only 450–500 mg/ $\mu$ l, within 15–20 min, they attained a maximum of 600 mg/ $\mu$ l and remained at this plateau level thereafter.

Flow rate does not appear to be an important factor in regulating levels of amylase or calcium under these conditions of stimulation. However, since the total volume of fluid secreted under the two conditions of stimulation were so different (Table I), it was probable that the volume of fluid would affect total output of calcium and amylase. Thus, calculations of total volume of saliva secreted during the 60 min of nerve stimulation were made, and are presented by the data in Table I. With stimulation of the auriculotemporal nerve, 653  $\pm$  103  $\mu$ l of fluid were secreted over the 60-min period when collection of saliva was continuously made. During the same interval, only 82  $\pm$  9  $\mu$ l were secreted when the sympathetic nerve was stimulated. Thus, there is an eightfold difference in volume when comparison between effects of the two conditions of stimulation are made. (These data agree very well with those of Young *et al.* (10) who showed that electrolyte concentrations of precursor fluids were similar under the two conditions of stimulation but the total volume produced was eight times greater with cholinergic than with adrenergic stimulation.)

Calculations of total output of calcium and

TABLE I. TOTAL OUTPUT OF AMYLASE, CALCIUM, AND FLUID IN RAT PAROTID SALIVA FOLLOWING STIMULATION OF THE AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM) FOR 60 MIN.\*

	Kind of stimulation	
	AT	SYM
Total volume ( $\mu$ l)	653 $\pm$ 103	82 $\pm$ 9
Total Ca output (nEq)	6744 $\pm$ 867	762 $\pm$ 91
Total amylase output (mg of reducing sub.)	8330 $\pm$ 906	41,076 $\pm$ 3337

\* Values are means  $\pm$  SE. The number of rats for each kind of stimulation was 7; output was continuously collected over 60 min. The differences between parasympathetic and sympathetic stimulation are statistically significant at a level of  $P < 0.001$ .

amylase were made. For example, since calcium concentrations under the two conditions of stimulation were generally similar in magnitude and in course of change whereas total volumes under the two conditions were markedly different, it was anticipated that total output of calcium with parasympathetic nerve stimulation would greatly exceed that obtained with stimulation of the sympathetic innervation. There was in fact about a ninefold difference in total calcium output when the two kinds of nerve stimulation were compared. This slight difference is attributable to the slight differences in calcium concentration observed between the two kinds of stimulation. Thus, a total of 6744  $\pm$  867 nEq of calcium were secreted with cholinergic stimulation and only 762  $\pm$  91 with sympathetic nerve stimulation. Similarly, amylase concentration of cholinergically-evoked saliva was very low (20 mg/ $\mu$ l) initially and throughout the period of collection, whereas the values with sympathetic nerve stimulation were 30–40 times greater. Again, the total output under the two conditions of nerve stimulation reflected these differences and the total amylase with stimulation of the sympathetic nerve was five times greater than that found with cholinergic stimulation, even though the total volume of cholinergically-evoked saliva was eight times greater.

Since flow rate was a modifying factor in assessment of total salivary output, it was necessary to relate the salivary output of these moieties to the levels remaining in the gland after stimulation was halted. From the data in Table II, it is clear that only a small (but

ally significant ( $P < 0.05$ )) change in concentration of the gland occurred after the auriculotemporal nerve was stimulated for 60 min. The change with sympathetic nerve stimulation was greater. Thus, a reduction in gland calcium was found after stimulation of the sympathetic nerve, but only a 13% decrease was observed when stimulation of the auriculotemporal nerve was employed. These changes were quite unexpected since the total output of calcium in surgically-evoked saliva was nine times greater than that found with sympathetic stimulation. The possibility that water loss in the gland could account for this apparent inconsistency was ruled out when analyses of calcium were made using dry weight of the gland. The percent changes were very different from those based on weight of the gland (Table II). It also seemed possible that with cholinergic stimulation, calcium changes in the gland might have occurred earlier than 60 min and, in fact, gland levels had been restored to control. Accordingly, calcium levels of the gland were assessed 20 min after initiation of stimulation. These values were virtually the same as those of controls (Table III).

Since calcium levels of saliva evoked by cholinergic and adrenergic nerve stimulation were so similar, there was the possibility that catecholamines were indeed released by stimulation of the cholinergic nerve, and that high calcium levels with either kind of stimulation must be attributed to adrenergic influences. However, this assumption was not found to be the case. The calcium levels of saliva evoked by cholinergic stimulation initiated 25 min after injection of both  $\alpha$ - and  $\beta$ -adrenergic blocking agents were not different from saliva levels of parasympathetically stimulated glands of rats that did not receive blocking agents (Table IV).

The depletion of gland levels of amylase paralleled the total output of the enzyme in the saliva. Thus, amylase levels of the gland were reduced by 23% after 60 min of stimulation of the cholinergic nerve; with sympathetic nerve stimulation there was a 46% reduction in gland levels.

**Discussion.** Present data strongly suggest that calcium and amylase secretion follow different routes with parasympathetic and sympathetic nerve stimulation. With auriculotemporal stimulation, calcium is transferred from plasma through the gland, with the

### II. CHANGE IN WATER CONTENT, AMYLASE ACTIVITY AND CALCIUM CONCENTRATION OF RAT PAROTID GLAND WITH STIMULATION OF EITHER AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM).<sup>a</sup>

Type of stimulation*	Rat parotid gland		
	Water content (percent)	Amylase activity (mg/mg wet wt)	[Ca] (mEq/kg wet wt)
Control	70.6 $\pm$ 0.8 (20)	537 $\pm$ 29 (11)	13.0 $\pm$ 0.3 (20)
AT.	74.3 $\pm$ 1.0 (8) ( $P < 0.05$ )	380 $\pm$ 24 (4) ( $P < 0.001$ )	11.3 $\pm$ 0.9 (8) ( $P < 0.05$ )
SYM.	78.0 $\pm$ 2.0 (5) ( $P < 0.05$ )	199 $\pm$ 16 (5) ( $P < 0.001$ )	8.4 $\pm$ 0.2 (5) ( $P < 0.001$ )

Values are means  $\pm$  SE; all values from experimental animals differ significantly from controls. \* In each case, period of stimulation was 60 min. The numbers in parentheses are number of rats.

### III. CHANGE IN WATER CONTENT, AMYLASE ACTIVITY AND CALCIUM CONCENTRATION OF RAT PAROTID GLAND FOLLOWING 20-MIN PERIOD OF STIMULATION OF EITHER THE AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM).<sup>a</sup>

Type of stimulation	Rat parotid gland		
	Water content (percent)	Amylase activity (mg/mg wet wt)	[Ca] (mEq/kg wet wt)
Control	70.6 $\pm$ 0.8 (20)	537 $\pm$ 29 (11)	13.0 $\pm$ 0.3 (20)
(20 min)	72.2 $\pm$ 0.7 (6) NS	434 $\pm$ 21 (6) ( $P < 0.01$ )	13.0 $\pm$ 1.0 (6) NS

Values are means  $\pm$  standard error. The numbers in parentheses are number of rats. NS = Not significantly ( $P > 0.05$ ).

TABLE IV. EFFECTS OF PRIOR ADMINISTRATION OF ADRENERGIC ANTAGONISTS ON CALCIUM OF SALIVA EVOKED BY STIMULATION OF THE AURICULOTEMPORAL NERVE (AT).<sup>a</sup>

Condition of stimulation	Ca concentration (mEq/l)
AT.	12.3 ± 0.76 (10)
AT. + IN.	12.8 ± 1.20 (6)
AT. + DI.	11.0 ± 0.58 (5)
AT. + DI. + IN.	11.3 ± 0.63 (4)

<sup>a</sup> Values are means ± SE. In no case did calcium values differ ( $P > 0.05$ ) from each other or from levels found with nerve stimulation alone. Propranolol (IN) (3 mg/kg) or dibenzyline (DI) (5 mg/kg) was administered ip. singly or together 25–40 min before resumption of stimulation of auriculotemporal nerve. The numbers in parentheses are number of rats.

levels in the saliva mainly representative of the amounts transferred through the glandular cells; thus a greater proportion of the total calcium output is not packaged and secreted with the amylase. Since the levels of amylase are low, the amounts of calcium packaged with the amylase are also low, and a large excess of calcium may be transferred independently from plasma to saliva. This is not the case with adrenergic nerve stimulation. With such stimulation, the amylase levels are very high and virtually all of the calcium may be packaged and secreted with the amylase (3). A parallelism between secretion of these two moieties with adrenergic but not cholinergic stimulation would be expected consequences (3), and the present data also supported the previous finding (3).

The principal finding that remains inexplicable is that a large output of calcium was observed with cholinergically stimulated saliva but little depletion of calcium in the gland was found. On the other hand, a small output of calcium was obtained with sympathetically induced secretion and there was a parallel between depletion of gland calcium and total output in saliva. Thus, secretory mechanisms for calcium secretion are not the same for both kinds of stimulation. The only explanation presently tenable to account for these differences is that uptake of calcium into the gland proceeds as rapidly as it is released into the saliva when cholinergic stimulation is employed. Furthermore, this uptake must occur very early or continuously, since gland depletion was very insignificant

in amount even as early as 20 min after stimulation was initiated.

Finally, the data show that the surprisingly high levels of calcium found with cholinergic nerve stimulation cannot be attributed to indirect effects of cholinergically-released catecholamines as postulated by Schramm's group (8) since the injection of both  $\alpha$ - and  $\beta$ -adrenergic antagonists prior to stimulation of the auriculotemporal nerve did not cause any modification in calcium levels from those observed with nerve stimulation alone. Furthermore, other evidence refutes the postulate that cholinergic stimulation involves catecholamine release. Thus, amylase activity of cholinergically evoked saliva samples is very low and the inhibition of adrenergic activity does not modify these levels further (2, 9, 11, 14, 17). In addition, when isoproterenol in concentrations (2.5  $\mu$ g/kg) too low to elicit secretion are injected during stimulation of the auriculotemporal nerve, a sharp increase in amylase, attributable to the isoproterenol, is observed (11). This clearly shows that the two different groups of receptors are involved in the amylase release and depend on kind of autonomic stimulation employed. Indeed, this view is further supported by work of several investigators (12, 14–16), since they have shown that cholinergic release of amylase is mediated through a pathway (cyclic GMP) separate from that of  $\beta$ -adrenergic release of amylase (cyclic AMP). Evidence is accumulating therefore that supports the thesis of separate pathways for the release of amylase and calcium, and this separation is determined by the kind of autonomic stimulation employed.

It is interesting that the total output of amylase induced by stimulation of the sympathetic nerve was fivefold greater than that of the parasympathetic nerve stimulation in spite of the finding that there was only a twofold difference in residual gland amylase. It is probable that stimulation of the sympathetic nerve enhances synthesis of amylase at a greater rate than that induced by stimulation of the parasympathetic nerve. However, the detailed mechanisms of enhancement of amylase synthesis evoked by the two kinds of autonomic nerve stimulation have not been clarified.

**Summary.** Calcium levels of rat parotid

evoked by stimulation of the auriculo-orbital nerve are high (11 mEq/l) and are higher than those evoked by stimulation of sympathetic innervation. Total calcium in the cholinergically-evoked saliva is very high but the depletion of gland calcium is insignificant 20 or even 60 min after initiation of stimulation. With sympathetic stimulation, there is a closer correlation between gland depletion and total output of calcium in the saliva. These findings suggest that the uptake mechanism for calcium with cholinergic stimulation is more rapid than that found with adrenergic stimulation. The levels of calcium in the cholinergically-evoked saliva are also not due to acetylcholine-induced release of catecholamines since calcium levels of cholinergically-evoked saliva are the same whether or not adrenergic stimulating agents are present. The total output of amylase in the saliva when sympathetic stimulation is employed is about five times greater than that found with cholinergic stimulation, and the reduction in gland amylase under these two conditions of stimulation is the same. The data also suggest that there is a parallelism between depletion of gland amylase and calcium and concentration and total output of these two substances in the saliva when adrenergic stimulation is used but that no parallelism between secretion of these substances is seen with cholinergic stimulation. It is suggested that with adrenergic stimulation all of the amylase is packaged together with calcium and the two are secreted together; however, with cholinergic stimulation, only a fraction of the total calcium is packaged with the amylase, and the remainder is transferred to the blood through the gland to the saliva.

Thus, two separate routes for secretion of calcium exist with cholinergic stimulation, and the pathways with the two kinds of nerve stimulation are different.

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# Temporal Changes in Ovarian Steroid-17 $\alpha$ -hydroxylase in Immature Rats Treated with Pregnant Mare's Serum Gonadotropin<sup>1</sup> (40375)

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Ovaries in immature rats increase their production of androgens and estrogens in response to pregnant mare's serum gonadotropin (PMS) but only after a lag period of several hours; in contrast, progesterone production increases within a few minutes (1-3). This delay suggests that the enzymes necessary for production of androgen and estrogen, i.e. 17 $\alpha$ -hydroxylase, 17-20 lyase, aromatase, and 17 $\beta$ -steroid dehydrogenase, must be induced by gonadotropic action. Indeed, Suzuki *et al.* (4) have demonstrated that the activity of each of these enzymes was increased in ovaries when measured 48 hr after exposure of immature rats to PMS.

Steroid 17 $\alpha$ -hydroxylase (EC 1.14.99.9) would appear to be an especially important enzyme in steroidogenesis because it could control androgen and estrogen production via either the 3-oxo-4-unsaturated pathway from progesterone or the 5-unsaturated pathway from pregnenolone. The latter pathway has been shown to predominate in the rabbit ovarian follicle (5) but for the rat the 4-unsaturated pathway may be preferred (4). The present study was undertaken to define the quantitative and temporal changes in hydroxylase of intact and hypophysectomized immature rats associated with exposure of the ovary to PMS. The results indicate that changes in this enzyme are related to secretory patterns of ovarian androgens and estrogens.

**Materials and methods.** Holtzman strain female rats were maintained in temperature (23  $\pm$  1 $^{\circ}$ ) and light (14 hr light: 10 hr dark) controlled quarters and given free access to water and Purina laboratory chow. In some experiments animals were injected with 2mg diethylstilbestrol (DES) dissolved in 0.1 ml of sesame seed oil on the 25th and 26th days of

age; controls received only oil. These animals were hypophysectomized via the parapharyngeal approach using ether anesthesia when they were 27 days old. After hypophysectomy a solution of 5% glucose was used for drinking water.

Pregnant mare's serum gonadotropin (PMS) (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.15 M NaCl and injected (20 IU in 0.2 ml, iv) on the 28th day of age. At various times after this injection animals, in groups of 10-15, were killed by decapitation; animals with incomplete hypophysectomy were discarded. Ovaries were removed, cleaned of adhering fat and oviduct, pooled, weighed and then homogenized (100mg wet wt/ml) in cold 0.15 M KCl. The homogenate was centrifuged for 20 min at 10,000g and then at 105,000g for 1 hr. The microsomal pellet from the latter centrifugation was resuspended in 0.15 M NaKPO<sub>4</sub> buffer (pH 7.4) and used for assay of 17-hydroxylase activity. The protein concentration of the microsomal suspension was determined using the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA). When in this dilution ( $\approx$ 1mg protein/ml) the hydroxylase is very labile and storage for 12 hr at -20 $^{\circ}$  removes more than half of its activity. In contrast, microsomal suspensions with protein concentrations of 10 mg/ml or greater retain activity for several weeks when stored at -20 $^{\circ}$ .

Hydroxylase activity was determined by the method of Kremers (6). This tritium exchange assay depends upon the reaction: 17 $\alpha$ <sup>3</sup>H-pregnenolone + NADPH + H<sup>+</sup> + O<sub>2</sub>  $\longrightarrow$  17 $\alpha$ -hydroxypregnenolone + <sup>3</sup>HOH + NADP<sup>+</sup>. Specifically labelled pregnenolone (15 mCi/mmol) was kindly prepared and characterized by Dr. P. Kremers (University of Liege, Belgium). Unlabelled pregnenolone (Sigma) was used to reduce the specific activity of the label to 2.4  $\mu$ Ci/ $\mu$ mole.

Generally 0.2 ml of the ovarian microsomal

<sup>1</sup>Supported in part by a grant from the National Institute of Aging.

nsion (1 ml = 100 mg wet weight of ) was incubated in a 20 ml glass scintillation vial. The medium (final volume = 1 ml) contained 100 or 200 nmole of  $17\alpha^3\text{H}$ -pregnenolone (0.25  $\mu\text{Ci}$ ), 0.5 mg tween 80 to solubilize the steroid, 5  $\mu\text{mole}$  glucose-6- $\text{PO}_4$ , 5  $\mu\text{mole}$  glucose-6- $\text{PO}_4$  dehydrogenase, 1  $\mu\text{mole}$   $\text{MgCl}_2$  and 0.7 ml  $\text{Na}_2\text{HPO}_4$  (pH 7.4); all chemicals were obtained from Sigma. Vials were incubated in a Dubnoff shaking water bath at  $37^\circ$ . Preliminary experiments had confirmed Kremers's (6) report that the enzyme activity is a function of incubation time, up to 60 min, the amount of ovarian homogenate incubated, 0.5 ml, and that 100 nmole of substrate stimulated the enzyme. In the present experiments the incubation time was 40 min.

The incubation was stopped by adding 1 ml of ice-cold distilled water followed quickly by 4 mg pellet of dextran-coated charcoal (Mallinckrodt Screening System Inc., North Hollywood, CA). We found this method as efficient as the addition of 20% trichloroacetic acid (6) but it has the advantage of removing the steroid radioactivity. The charcoal was separated by centrifugation at 2000g for 30 min. The supernatant was transferred to a 25  $\times$  100 mm glass tube and the water distilled under reduced pressure at a temperature of  $40^\circ$ . A 1 ml aliquot of the distillate was placed in a scintillation vial along with 10 ml of scintillant (Packard Instrument Co., Downers Grove, IL). The mixture was counted in a Beckman Scintillation counter with an efficiency of 64% for tritium. The enzyme activity was expressed as nmole of pregnenolone converted per mg protein per hour. In all experiments the homogenates were incubated in duplicate and in some cases at two dose levels; the series was repeated. The details for spectral analyses of enzyme determinations were done using Student's *t* test:  $p < .05$  was considered significant.

**Results.** Ovaries of intact immature rats showed considerable 17-hydroxylase activity (Table 1). Two groups of animals injected with PMS and killed 48 or 96 hr later had enzyme activities which were not significantly different from that of starting controls. Eight hours after injection of 20 IU PMS the activity was reduced by about 90%. The enzyme level

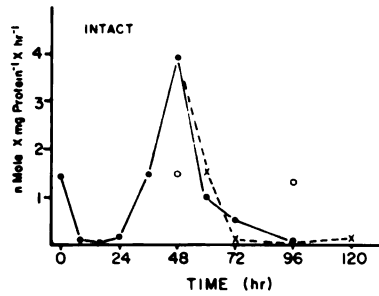


FIG. 1.  $17\alpha$ -hydroxylase activity, determined by a tritium exchange assay, in the ovaries of immature (28 day) rats. PMS(20 IU) was injected (iv) at time 0. Groups of 10–15 rats were killed at various times, the ovaries pooled, homogenized and centrifuged. The 105,000g pellet from the homogenate was incubated for 40 min with  $17\alpha^3\text{H}$ -pregnenolone and the tritiated water produced distilled under reduced pressure; the enzyme activity is expressed as nmole of substrate converted per hour per mg protein. Each point represents the mean activity in at least 6 samples; SEM does not exceed the area covered by the symbols. Solid circles (●) indicate values for animals given PMS; single points (○) indicate enzyme activity for ovaries from control animals given 0.2 ml normal saline. The dotted line indicates changes in enzyme activity in ovaries from animals injected with PMS at time 0 and 10 IU human chorionic gonadotropin (sc) at 48 hr to insure ovulation in all animals.

remained low through 24 hr and then began an increase so that by 36 hr the activity was the same as that found in starting controls. The enzyme activity continued to increase to a peak level at 48 hr, but it then decreased drastically during the next 12 hr. Activity continued to decrease to almost undetectable levels by 72 hr. Histological examination of the ovaries at 48 and 60 hr revealed many large antral follicles and a highly stimulated theca and interstitium but little luteinization of granulosa. To insure ovulation and luteinization animals were given (sc) 10 IU of human chorionic gonadotropin (hCG) (Antuitrin-“S”, Parke-Davis & Co., Detroit, Mich) 48 hr after the injection of PMS. The ovaries were assayed 12, 24, 48 and 72 hr after the hCG (Fig. 1). This treatment did not alter the pattern of decline in hydroxylase activity to any extent.

Ovarian hydroxylase levels in hypophysectomized animals are shown in Fig. 2. The starting controls (24 hr posthypophysectomy) had an activity which was 31% less than that found in uninjected intact females ( $1.42 \pm$

0.03 vs  $0.98 \pm 0.03$  nmole  $\times$  mg protein $^{-1} \times$  hr $^{-1}$ ). In an additional 24 hr the activity in hypophysectomized animals declined to  $0.76 \pm 0.08$  which indicated a decay rate or half-life of more than 48 hr when endogenous gonadotropins were removed.

Ovaries in rats given DES were 36% heavier at the time of PMS administration than those given oil; the increase was due to larger numbers of granulosa cells in the DES-treated animals. However, hydroxylase activity in the enlarged ovaries was only 4% of the level found in oil-treated animals. The enzyme activity increased in the ovaries of both oil and DES-treated animals between 12 and 24 hr after PMS. In the oil-treated animals enzyme activity had returned to the preinjection control level by 24 hr, remained at this level until 36 hr and then increased sevenfold in the next 12 hr (Fig. 2). Hydroxylase also increased in the DES-treated animals but to a somewhat lesser extent. However, even in the latter, enzyme activity exceeded that found at 48 hr in intact immature females. The enzyme activity of ovaries in hypophysectomized oil-treated animals decreased 27% between 60 and 72 hr (NS  $P > .05$ ) after PMS

but it was still 63% higher than the peak level found in intact animals at 48 hr.

**Discussion.** The reason for the initial decrease in hydroxylase activity in the ovaries of intact or oil-treated hypophysectomized animals after the injection of PMS is unknown. A similar phenomenon occurs in testicular hydroxylase after administration of hCG (7). The drop in enzyme activity is too rapid (90% in 8 hr) to be accounted for by inhibition of further production and suggests that some other process of inactivation is involved. Testicular hydroxylase has a half-life of 2.5 days (7); the ovarian enzyme seems to disappear at about the same rate when gonadotropin is removed.

Attention has recently been focused again upon the kinds of cells which contain 17-hydroxylase (8). Presumably this enzyme is largely, if not entirely, restricted to thecal and interstitial tissues of the mammalian ovary (8, 9). The present results are consistent with this view; that is, ovaries which were increased in size and weight by increasing the number of granulosa cells with DES did not have a proportionate increase in hydroxylase activity (Fig. 2). We must be careful in interpreting these results however because the ovaries were exposed to high levels of a potent estrogen and this may have had an effect upon hydroxylase or upon the cellular response to gonadotropin. Some suggestion of such an effect, although not necessarily upon granulosa, is gained from the finding that the enzyme level at time 'O' in animals given DES was very low; the enzyme would not be expected to disappear this quickly in animals lacking a pituitary.

If the granulosa cell does not have hydroxylase then luteinization would not be expected to increase the enzyme level in the ovary. Actually luteinization was associated with a drastic decrease in hydroxylase (Fig. 1); as with the initial decrease this one also appeared to be due to an inactivation process considering the rate of decrease. This decrease in hydroxylase may be causally related to a decrease in ovarian androgen and estrogen secretion seen between 48 and 60 hr after PMS administration to immature rats (10, 11). A similar decrease is found with the LH surge on proestrus in the rat and has prompted several speculations into possible

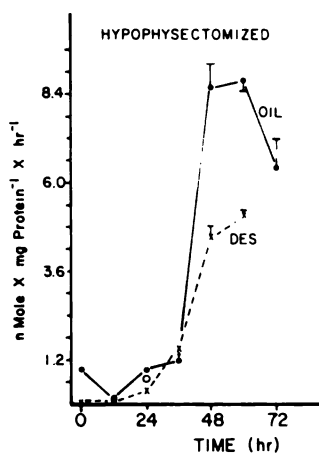


FIG. 2. Hydroxylase activity in the ovaries of hypophysectomized animals; methods indicated in legend for Fig. 1. Animals were injected with 0.1 ml of sesame seed oil (●—●) or 2mg diethylstilbestrol (DES) (x—x) on the 25th and 26th days of age; hypophysectomy was performed on day 27 and PMS was injected 24 hr later. The vertical line at each point indicates the SEM for at least 6 samples. The single point (○) indicates the enzyme level in a group pretreated with oil and injected with normal saline.

mechanisms. While intra-ovarian autoregulation by steroids or their metabolites has received the most attention (see ref in 11) Katz and Armstrong (12) suggested that LH caused a decrease in aromatase and this was responsible for the drop in estrogen production. The surge in LH associated with PMS treatment may also be responsible for the decrease in hydroxylase found in the intact animals of present study. Further support for this is obtained from the finding that injection of 10 IU hCG into hypophysectomized rats 48 hr after giving PMS resulted in a 90+% decrease in hydroxylase activity within 12 hr (unpublished data). However, this may not be the only factor involved since prolactin caused a 70% reduction in the enzyme activity stimulated by PMS and a 94% reduction in that stimulated by hCG in hypophysectomized animals (unpublished data). Considerably more study is needed for clarification of the control of 17-hydroxylase.

The increase in hydroxylase activity found in hypophysectomized animals coincides with an increase in serum estradiol and testosterone (1, 2). However, the amounts of these steroids in DES or oil-treated animals does not correlate with the enzyme levels found; DES-treated animals had significantly more estrogen and testosterone than did oil-treated controls (3). Perhaps the enzyme levels found in DES-treated animals are inaccurate due to the large volume of granulosa present which does not contribute to enzyme activity, or equally likely, the amount of enzyme present may not indicate the amount of function possible.

**Summary.** Steroid 17 $\alpha$ -hydroxylase was measured, using a tritium exchange assay, in the microsomal fraction of ovaries from immature intact or hypophysectomized rats exposed to 20 IU pregnant mare's serum gonadotropin. The hypophysectomized animals were pretreated with diethylstilbestrol (DES) to increase the ratio of granulosa:theca + interstitium in the ovary; controls received oil vehicle. In intact animals hydroxylase levels decreased within 8 hr after injecting PMS but

by 48 hr the concentration was more than 3 times that found in starting controls; after 48 hr the enzyme level decreased drastically and remained low through 120 hr. In oil-treated hypophysectomized rats hydroxylase activity decreased within 12 hr after PMS but in DES-treated animals the enzyme was already extremely low. In both, the enzyme level reached much higher levels than in intact animals and it did not decrease significantly through 72 hr after PMS. The results indicate that 17-hydroxylase activity is induced by PMS treatment but that the enzyme is actively destroyed beginning at 48 hr in intact animals; this could account for the decrease in estrogen and androgen production associated with the ovulatory surge in gonadotropins which occurs on the second day after PMS injection.

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Curie	Ci	molar (concentration)	<i>M</i>
degree Celsius (Centigrade)	-°	mole	spell out
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diameter	diam	nanogram	ng
gram	g	nanometer	nm
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inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
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kilogram	kg	revolutions per minute	rpm
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